

#843R2 - BARGER

1003541802

#926 - BHAGAT

1003541811

will be assessed initially by periodic measurements of serum cholesterol values. Results from initial experiments will later be used to determine the amounts of cholesterol, saturated fats, and concentrations of carbon monoxide that should be tested systematically for their effects on aortic atherosclerosis in the squirrel monkey.

1003541810

8. Brief statement of working hypothesis:

2.

See proposal

9. Details of experimental design and procedures (append extra pages as necessary)

See proposal

1003541814

Appendix I.

1. Names of investigators including titles and degrees:

- A. C. Barger, M.D. Robert Henry Pfeiffer Professor of Physiology
- P. B. Dews, M.B., Ch.B., Ph.D. Stanley Cobb Professor of Psychiatry
and Psychobiology
- K. C. Hayes, D.V.M., Ph.D. Assistant Professor of Nutrition in the
School of Public Health
- J. A. Herd, M.D. Associate Professor of Physiology
- R. T. Kelleher, Ph.D. Professor of Psychobiology in the Department
of Psychiatry
- W. H. Morse, Ph.D. Associate Professor of Psychobiology in the
Department of Psychiatry
- R. Beeuwkes, III, Ph.D. Assistant Professor of Physiology
- L. D. Byrd, Ph.D. Instructor in Psychobiology in the Department of
Psychiatry.
- S. R. Goldberg, Ph.D. Research Fellow in Psychobiology in the
Department of Psychiatry.
- N. P. Westmoreland, D.V.M., Ph.D. Assistant Professor of Nutrition
in the School of Public Health
- S. A. Grose, B.S. Research Associate in Psychobiology in the
Department of Physiology
- N. R. Leclair, M.S.E. Electronic Engineer

1003541808

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

July 31, 1973

Grant application 843R2
CARDIOVASCULAR

To: The committee comprising Drs. Bing, Meier and Sommers

Subject: A. Clifford Barger, M.D., Harvard Medical School
2nd Renewal Application #843R2
"Behavioral Hypertension and Arteriosclerosis: Effects of
Nicotine and Carbon Monoxide"

History

A three-year plan was approved to start 1972, at no more than \$50,000. per year.

Application #843R2 requests (for the first time in the history of this grant) no more than the \$50,000. per year level established earlier.

Documents Submitted (attached)

1. Application dated July 19, 1973 with Appendices I and II.
2. Progress Report #2 (7/1/72 to 6/30/73) submitted as Appendix III.

FWN:gh

FWN
F.W.N.

Attachment

1003541803

13. Budget for the coming year: see Appendix IV.

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount
(including fringe benefits)

A.C. Barger

15

None

P.B. Dews

15

None

J.A. Herd

25

None

R.T. Kelleher

25

None

W.H. Morse

25

None

Technical

W. Goulding, Research Assistant I

100

9,094

S.A. Grose, Research Associate

100

14,623

L. King, Research Assistant II

100

12,310

Sub-Total for A 36,027

B. Consumable supplies (by major categories)

Animal purchase, care, food and supplies

3,451

Pathology

500

Electrical supplies

1,000

Physiological supplies

1,000

Surgical supplies

500

Sub-Total for B 6,451

C. Other expenses (itemize)

Art work, photography and publication costs

Sub-Total for C 1,000Running Total of A + B + C 43,478

D. Permanent equipment (itemize)

None

Sub-Total for D -

E

6,522

E. Indirect costs (15% of A+B+C)

\$50,000.00

1003541806

8. Any additional facilities now required? Describe briefly:

None.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

None.

10. Append outline of experimental protocol for ensuing year. Appendix II.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

None.

1003541805

12. Summary progress report (append in standard form as separate document, unless recently submitted). Appendix III.

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

July 27, 1973

Grant Application No. 926
CARDIOVASCULAR

To: The committee comprising Drs. Bing, Jacobson and
Sommers

Subject: Budh Dev Bhagat, Ph.D., St. Louis University, Missouri
New application No. 926
"Effect of Smoking on the Cardiovascular System in
Experimental Hypertension"

History

Grant No. 588, with renewals and continuations, supported Bhagat's studies of nicotine effect on biogenic amines in the central nervous system from 1966 through 1973. A request for further continuation of support was denied by SAB in March 1973.

Application No. 926 (on a somewhat different topic) requests \$31,360 plus two additional years.

Documents Submitted (attached)

1. Application dated July 19, 1973 (5 pages).
2. "PROPOSAL" (undated) 13 pages. This appears to be a copy of a NIH application.

Comment

Staff comment may follow soon.

F.W.N.
F.W.N.

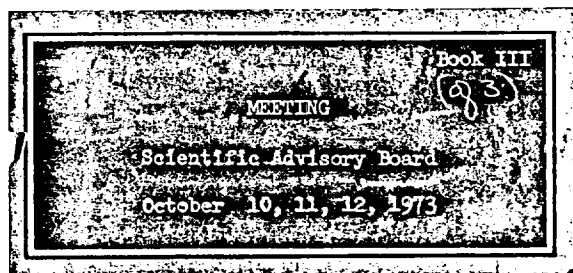
FWN:wg
Encls.

1003541812

FIVE PERTINENT PUBLICATIONS

1. B. Bhagat. Effects of dermic administration of nicotine and storage and synthesis of noradrenaline in rat brain. Br. J. Pharmac. 38: 86-92, 1970.
2. B. Bhagat. Influence of chronic administration of nicotine on the turnover and metabolism of noradrenaline in the rat brain. Psychopharmacologia 18: 325-332, 1970.
3. B. Bhagat, T. Bayer and C. Lind. Effect of chronic administration of nicotine on drug-induced hypnosis in mice. Psychopharmacologia 21: 287-293, 1971.
4. B. Bhagat and M.W. Rana. Effect of chronic administration of nicotine on the concentrations of adrenal enzymes involved in the synthesis and metabolism of adrenaline. Br. J. Pharmac. 43: 250-251, 1971.
5. Ping-lung Chang, B. Bhagat and John J. Taylor. Effect of chronic administration of nicotine on acetylcholinesterase activity in the hypothalamus and medulla oblongata of the rat brain. An ultrastructural study. Brain Res. 54: 75-84, 1973.

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Space: Our laboratory and office space (approximately 600 sq.ft.) is well equipped with all the standard facilities. In addition, a cold room radioisotope room, animal room and machine shop are also available.

Equipment: In addition to standard laboratory equipment, such as glassware and other apparatus, the following items are available for our use: Grass stimulator, spectrofluorometer (Aminco-Bowman), mechanical shaker, and International portable refrigerated centrifuge.

In addition, facilities of the Physiology Laboratory of the Department of Gynecology and Obstetrics (Dr. Cavanagh) will be available for use. The facilities are adequate for the sterile operative procedures required.

Animal Research Space: Ample animal and laboratory space is available in the new renovated Animal Care Facility at St. Louis University Medical Center to permit proper conduct of these studies. These quarters are under the direction of a veterinarian who quarantines and conditions animals prior to use in experiments.

Library: An excellent medical library supports the research service. It includes over 5,000 volumes and regularly subscribes to 189 scientific periodicals. An excellent interlibrary loan system with the four Universities and two medical societies in our area gives us ready reference material promptly. The Yalem Computer Center of St. Louis University is readily available for the processing of data and gives a priority to medical research.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See page attached

13. Publications: (five most recent and pertinent of investigator(s), append list, and provide reprints if available).

1003541815

PROPOSAL

Budh D. Bhagat

1003541820

7-843A2

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

JUL 30 1973

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐

Second Renewal ☒

Date: July 19, 1973

1. Principal Investigator (give title and degrees): Appendix I.

A. Clifford Barger, M.D.

Robert Henry Pfeiffer Professor of Physiology

J. Alan Herd, M.D.

Associate Professor of Physiology

2. Institution & address:

Harvard Medical School

25 Shattuck Street

Boston, Massachusetts 02115

3. Department(s) where research will be done or collaboration provided:

Department of Physiology, Harvard Medical School

Psychobiology Laboratory, Department of Psychiatry, Harvard Medical School

Department of Nutrition, Harvard School of Public Health

4. Short title of study:

Behavioral Hypertension and Arteriosclerosis: Effects of Nicotine and Carbon Monoxide

5. Proposed renewal date:

January 1, 1974

6. How results to date have changed earlier specific research aims:

No change in specific Research Aims.

Specific aims of this research program are to determine the effects of nicotine and carbon monoxide on behavioral performances, heart rate, arterial blood pressure, serum cholesterol, and atherosclerosis in the squirrel monkey.

7. How results to date have changed earlier working hypothesis:

No change in Working Hypothesis

(a) Nicotine administered in small amounts over long periods of time suppresses cardiovascular responses to certain behavioral procedures, and

(b) Carbon monoxide administered in low concentrations over long periods of time has inconsequential effects on long term hypertensive and arteriosclerotic response to certain behavioral procedures and atherogenic diets.

1003541804

Electron Microscopy

Appropriate sections from heart, lung, liver, kidneys, endocrine glands will be fixed at specified times into 3% glutaraldehyde for one hour followed by Dalton's osmium fixative for one hour. The tissues will be embedded in Epon, sectioned and stained with uranyl acetate and lead citrate. Electronmicroscopy will be made with the use of RCA EMU 3 G Electronmicroscope III.

Analysis of Data

We have on the premises of the Department a D.E.C.-LINC computer for statistical analysis of the data. We will have a part-time computer programmer in the Department and if the need for special data analysis develops or if new programs are needed, he will be available to write and develop such programs. In this regard, it is anticipated he will be extremely helpful in developing programs to analyze the numerous samplings of the transmembrane potential before and after drug administration.

1003541833

4.

14. First year budget:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)
even if no salary requested).

B. Bhagat, Ph.D.

20%

none

1 Post-doctoral fellow

100%

(to be employed)

REDACTED

Technical

1 Technician (to be employed)

100%

Animal Caretaker

50%

REDACTED

Fringe Benefits

Sub-Total for A

REDACTED

B. Consumable supplies (by major categories)

Radioactive material

1,000.

Drugs and chemicals

1,000.

Animals

2,500.

Maintenance of equipment

400.

Sub-Total for B

4,900.

C. Other expenses (itemize)

Travel to attend National meetings

400.

Photographic material

200.

Reprint costs

200.

Sub-Total for C

800.

Running Total of A + B + C

REDACTED

D. Permanent equipment (itemize)

NONE

Sub-Total for D

E

3,930.

E. Indirect costs (15% of A+B+C)

(exclusive of fringe benefits)

Total request

REDACTED

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	4,900.	800.	---	4,072.	REDACTED
Year 3		4,900.	800.	---	4,233.	

1003541818

ek
207

CURRICULUM VITAE

BUDH DEV BHAGAT, Ph.D.

Born: India, January 1, 1926; U.S. Citizen

Education:

Ph.D., Pharmacology, (Faculty Medicine),
London University, 1961

Postdoctoral in Pharmacology,
University of Wisconsin Medical School, 1962

Postdoctoral in Pharmacology,
University of Minnesota Medical School, 1963

Faculty Appointments:

Assistant Professor, Department of Pharmacology,
Howard University Medical School, 1964-66

Assistant Professor, Department of Pharmacology,
New York Medical College, 1966-68

Associate Professor, Department of Physiology
Associate Professor, Department of Pharmacology,
St. Louis University School of Medicine, 1968-71

Professor, Department of Physiology
Professor, Department of Pharmacology,
St. Louis University School of Medicine, 1971-

Major Research Interests:

Autonomic Nervous System, Neurotransmitter, Cardiovascular

Committee Appointment:

Member - Advisory Board for "Neurosciences Research," Academic Press

Publications:

Approximately 169 publications to date.

1003541816

SUMMARY

Cigarette smoking is suggested to be one of the major hazards in the United States. It is implicated in cardiovascular diseases. The present proposal is to conduct a study to throw light on the mechanism of action of smoking on the cardiovascular system in experimental hypertension. For this purpose we will measure endogenous catecholamines, accumulation of ^3H -norepinephrine, the activity of enzymes involved in synthesis and degradation, and the turnover rate of norepinephrine in the heart, adrenal glands, and blood vessels, and determine the reactivity, in vitro, of the vascular smooth muscle. These studies will be conducted not only during smoking, but also during periods of withdrawal from cigarette smoking. An attempt will be made to correlate the biochemical changes in the cardiovascular system with the onset and degree of initial and subsequent hypertension. Finally, the effects of various drugs on hypertension will be determined.

It is our belief that this combined physiological and biochemical study may elucidate the role of smoking in the acceleration of cardiovascular diseases.

1003541821

#926

CARDIOVASCULAR

Comm.

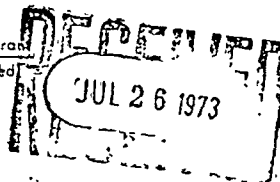
Dr. Ring
Dr. Jacobson
Dr. Sommers

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)

Date: July 19, 1973



1. Principal investigator (give title and degrees)

Budh Dev Bhagat, Ph.D.
Professor of Physiology

2. Institution & address

St. Louis University School of Medicine
1402 South Grand Boulevard
St. Louis, Missouri 63104

3. Department(s) where research will be done or collaboration provided:

Department of Physiology

4. Short title of study

Effect of Smoking on the Cardiovascular System in Experimental Hypertension

5. Proposed starting date October 1, 1973

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

See proposal

1003541813

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

July 6, 1973

Grant application No. 918

TO: The committee comprising Drs. Bing, Jacobson and Wyatt

SUBJECT: H. Fred Downey, Ph.D., University of Texas, Dallas
New application No. 918
"Effects of Tobacco Smoke and Nicotine on Coronary Collateral
Blood Flow"

History

This proposal was case #150 and application was encouraged.

Application #918 requests \$15,290 plus one additional year.

Document Submitted

Attached is application dated June 14, 1973.

FWN:gh

Encl.

FWN
F.W.N.

1003541836

1003541835

#918 - DOWNNEY

10. Forman, R., E. S. Kirk, J. M. Downey, and E. H. Sonnenblick. Nitroglycerin and heterogeneity of myocardial blood flow. Reduced subendocardial blood flow and ventricular contractile force. J. Clin. Invest. 52: 905-911, 1973.
11. Hammond, E. C. Smoking in relation to the death rates of one million men and women, in Haenszel, W., editor, Epidemiological approaches to the study of cancer and other diseases, Bethesda, United States Public Health Service, National Cancer Institute, Monograph No. 19, January, 1966, pp. 127-204.
12. Kannel, W. B., W. P. Castelli, and P. M. McNamara. The coronary profile: 12-year follow-up in the Framingham study. J. Occup. Med. 9: 611, 1967.
13. Kattus, A. A., and D. E. Gregg. Some determinants of coronary collateral blood flow in the open-chest dog. Circ. Res. 7: 628-642, 1959.
14. Leb, G., F. Derntl, E. Robin, and R. J. Bing. The effect of nicotine on effective and total coronary blood flow in the anesthetized closed-chest dog. J. Pharmacol. Exp. Ther. 173(1): 138-144, 1970.
15. Mathes, P., and J. Rival. The effect of nicotine on regional blood flow in the canine heart. Proc. Soc. Exp. Biol. Med. 138: 361-364, 1971.
16. Mulcahy, R., N. J. Hickey, and B. J. Maurer. Coronary heart disease in women. Study of risk factors in 100 patients less than 60 years of age. Circulation 36: 577, 1967.
17. Schaper, W. The Collateral Circulation of the Heart. American Elsevier, New York, 1971.
18. Travell, J., S. H. Rinzler, and D. Karp. Cardiac effects of nicotine in the rabbit with experimental coronary atherosclerosis. Ann. N.Y. Acad. Sci. 90: 290-301, 1960.
19. West, J. W., S. V. Guzmán, and S. Bellet. Cardiac effects of intracoronary arterial injection of nicotine. Circ. Res. 6: 389-395, 1958.

1003541840

3
tension in response to injection of 5 mg/kg of norepinephrine (DeQuattro et al. *Cir. Res.* 24: 545, 1968) have been reported

To determine whether onset and degree of initial and subsequent hypertension are augmented by smoking in experimental hypertension.

To determine whether or not the changes in the catecholamine pattern are related to any changes in function of the components in the cardiovascular system.

It has been found that in the heart of SHR, norepinephrine turns over

1003541824

The following parameters will be measured in the adrenal gland: 1) norepinephrine, 2) epinephrine, 3) TH activity, 4) MAO activity, 5) COMT activity, 6) phenylethanol-N-methyl transferase.

The following parameters will be measured in superior cervical ganglia: 1) norepinephrine, 2) TH activity, 3) MAO activity, 4) COMT activity.

The following parameters will be measured in heart and vascular tissues: 1) norepinephrine, 2) capacity to take up and accumulate ^3H -norepinephrine, 3) the rate of metabolism of ^3H -norepinephrine, 4) rate of conversion of ^3H -tyrosine to ^3H -norepinephrine, 5) MAO activity, 6) COMT activity, 7) TH activity.

All vascular tissues will be carefully cleaned of adhering tissue with forceps or a small nylon brush as described by Koletsky et al (Proc. Soc. Exp. Biol. Med. 102: 12-15, 1959). Microscopic examination of the vessels will be made to confirm that adhering tissues (connective tissue, fat and extravascular nerves) have been removed.

Histomorphological Changes: Tissues such as kidney, lungs, liver, heart, endocrine glands from experimental animals will be studied histologically using H & E and PAS stains; also ultrastructure of these tissues will be studied.

These studies will be carried out with collaboration of Dr. K. Christensen, Professor of Anatomy.

1003541829

8. Any additional facilities now required? Describe briefly:

None

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

See Page 3, 13A, Technical

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

Influence of Nicotine on Experimental Atherosclerosis and Its Determinants,
by Edwin R. Fisher, M.D., R. Rothstein, M.S., Mark H. Wholey, M.D., and R. Nelson, M.S.
Archives of Pathology. In press.

1003541854

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Specialized Center for Research in Hypertension (SCOR)	USPHS HL 14150	\$1,534,351.	6.1.71-5.31.76
Kidney Function in Experi- mental Heart Failure	USPHS HL 02493	149,000.	9.1.69-8.31.74
Basic Types of Effects of Drugs on Behavior	USPHS MH 02094	105,141.	12.1.70-11.30.75
Central Control of Distribution of Organ Blood Flow	USPHS HL 09154	45,148.	9.1.72-8.31.75
Effects of Drugs on Reactions to Aversive Stimuli	USPHS MH 07658	219,126.	5.1.70-4.30.75
Biotechnology Resource in Electronprobe Microanalysis	USPHS 1 P07-BR00679 R01-HL15552 PENDING OR PLANNED	804,069.	6.26.72-8.31.77

None

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to

President and Fellows of Harvard College

Mailing address for check:

25 Shattuck Street

Boston, Massachusetts 02115

Principal investigator

Typed Name A. Clifford BargerSignature A. Clifford Barger Date 7/24/73Telephone 617 734-3300 486
Area Code Number Extension

Responsible officer of institution

Typed Name Henry C. MeadowTitle Executive Secretary, Committee on ResearchSignature Henry C. Meadow & Development Date 7/24/73Telephone 617 734-3300 441
Area Code Number Extension

1003541807

6.
EDWIN R. FISHER, M.D.

PROGRESS REPORT NO. 2 (Continued)

information concerning the lungs in such animals. Therefore, in addition to the relatively short term observations of 2-3 months as indicated in the original protocol, some animals will be subjected to the effects of smoking for 9-12 months. Indeed, some animals have already been sacrificed after 10 months of cigarette smoking. Although the number in this category at present are relatively few, nevertheless preliminary study has failed to disclose significant cardiovascular or pulmonary alterations related to such treatment.

Aside from the extended period of observation and examination of the lungs in animals subjected to cigarette smoking, it is our intention to adhere to the protocol as originally submitted.

1003541858

#039R2 -- FISHER

1003541851

16. Other sources of financial support.

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
NONE			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
NONE			

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made"

Checks payable to

Louis Univ. School of Medicine

Mailing address for checks

Controller, 221 N. Grand

St. Louis, Missouri 63103

Principal investigator

Typed Name Budh D. Bhagat

Signature B. Bhagat Date 7/23/73

Telephone (314) 664-9800 412
Area Code Number Extension

Responsible officer of institution

Typed Name George E. Thomas

Title Asst. Vice Pres. & Research Administrator

Signature G. E. Thomas Date 7/23/73

Telephone (314) 865-2288 541
Area Code Number Extension

1003541819

JUSTIFICATION OF BUDGET:

Personnel

Technical assistance is required for animal preparation, sample processing and data collection and analysis. The complex nature of the animal preparation, the use of radioactive isotopes, the operation of such instruments as the blood flowmeter, gamma counter, and laboratory computer require the skills and training of a Research Technician. Mr. Williams is presently employed in our laboratory and is familiar with the procedures to be used in this investigation.

Supplies

Radioactive microspheres with diameters between 8 and 10 microns are available on special order from the 3-M Company. The cost is \$750 per 1 mc and \$825 for 2 mc. We will require two shipments of 2 mc each of three differently labeled microspheres. By combining our orders with orders of Dr. David Fixler of the University of Texas Southwestern Medical School, the cost of the microspheres can be considerably reduced. The amount budgeted, \$1,650, should cover the cost of microspheres for this investigation. Although microspheres of larger diameter are available at lower cost, they tend to overestimate subendocardial flow. The greater mass and specific gravity of the larger microspheres appear to prevent them from making sharp turns out of the penetrating arteries and, thus, divert them to the endocardial tissue (Domenech *et al.*, *Circulation Res.* 25: 581-596, 1969). However, the 8-10 μ microspheres do not exhibit this tendency to overestimate subendocardial flow (Buckberg *et al.*, *Circulation Res.* 30: 67-81, 1972). Since measurement of the transmural distribution of coronary collateral flow is a vital part of this investigation, we feel justified in requesting the funds necessary to use the most accurate means of making this measurement, the 8-10 μ microspheres.

1003541849

used for assay of monoamine oxidase activity. The remaining homogenate will be centrifuged at 26000 g for 20 min. Aliquots of the clear supernatant fluid will be assayed for tyrosine hydroxylase, PNMT and COMT activities.

Monoamine oxidase activity will be assayed by measuring the conversion of ^{14}C -tryptamine to ^{14}C -indoleacetic acid as described by Wurtman and Axelrod (Biochem. Pharmacol. 12: 1439, 1964).

Catechol-o-methyl transferase (COMT) will be assayed by measuring the formation of ^{14}C -metanephrine on incubation with (-) epinephrine and ^{14}C -methyl-s-adenosylmethionine as described by Axelrod (in Method of Enzymology, Vol. 5, p. 748, 1959, New York Acad. Press).

Tyrosine hydroxylase activity will be assayed by the method of Levitt et al (J.P.E.T. 148: 1, 1965) with modifications described by Mueller et al (J.P.E.T. 101: 379, 1969).

Phenylethanol-N-methyl transferase activity will be assayed by the method of Axelrod (J. Biol. Chem. 237: 1657, 1962) using normetanephrine as the substrate and ^{14}C -S-adenosylmethionine will serve as a methyl donor.

Synthesis of norepinephrine in isolated tissues

The measurement of norepinephrine turn-over rate will be made by the amount of ^3H -norepinephrine formed from the ^3H -tyrosine according to the method of Weiner and Rabadjiya (J. Pharmacol. Exp. Ther. 160: 61-71, 1968).

Many of these methods are already operative in our laboratory. The others will be set up for the purposes of this investigation.

Morphological Investigation

Complete autopsy will be performed in all experimental animals. In addition to a general survey of the histopathological changes of individual organs, and special attention will be paid in the study of the vascular changes in the heart, lung, kidneys, liver and endocrine glands.

Representative blocks of tissues from each organ will be placed into 10% neutral buffered formalin, Carnoy's fluid, and 100% ethanol respectively in order to carry out appropriate special stains in addition to a routine hematoxylin and eosin stains. The special stains utilized will include: Mallory's Azan stain, Periodic Acid-Schiff reaction, Verhoeff Van Gieson stain and phosphotungstic acid hematoxylin stain. Appropriate blocks of tissues will also be frozen immediately to perform various enzyme stains.

1003541832

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Edwin R. Fisher, M.D.

35

Mark Wholey, M.D.

10

Technical

Marie Tomko (Histotechnician)

85

7620

Virginia Malek (EM Technician)

85

6000

Dolores Van Holt (Histotechnician)

25

2000

Yang ksien Ke, Ph.D. (Chief, Experimental Path.)

25

2000

Sub-Total for A

17620

B. Consumable supplies (by major categories)

Animals

1000

Histopath supplies

500

Electron microscopy supplies

750

Radiologic supplies

750

Drugs (Cholesterol)

500

Sub-Total for B

3500

C. Other expenses (itemize)

Publication

200

Reference Cigarettes

400

Sub-Total for C

600

Running Total of A + B + C 21,720

D. Permanent equipment (itemize)

None

Sub-Total for D

E. Indirect costs (15% of A+B+C)

E

3258

12. Biographical sketch of collaborator:

Paul E. Parker, Ph.D.

BIRTHPLACE: **REDACTED**

EDUCATION:

University of Texas, Austin 9/63-5/64 Major field -
Biology

Southern Methodist University, Dallas 9/64-5/67 B.S. -
Biology

North Texas State University, Denton 9/67-8/69 M.S. -
Physiology

Michigan State University, East Lansing 9/69-10/72 Ph.D. -
Physiology

POSITIONS HELD:

North Texas State University - Laboratory Instructor, Biology,
9/67 - 8/69

North Texas State University - Graduate Research Assistant,
Biology, 9/68 - 8/69

Michigan State University - Predoctoral Fellow, Physiology,
9/69 - 10/72

Michigan State University - Post-doctoral Fellow, Physiology,
11/72 - Present

University of Texas Southwestern Medical School - Post-doctoral
Fellow, Physiology, To be appointed July 1, 1973

ACADEMIC AND PROFESSIONAL HONORS:

NIH Predoctoral Traineeship, September, 1969 to October, 1972.

PROFESSIONAL SOCIETIES AND RELATED ORGANIZATIONS:

REDACTED

1003541846

7. Effect of Chemical Sympathectomy on Development of Hypertension:

Most of the investigators believe that excessive activity of the sympathetic nervous system contributes to the development and persistence of abnormal arterial pressure in patients with primary hypertension. It is therefore planned to study the effect of smoking on development of hypertension in rats pretreated with 6-OH dopamine which causes a long-lasting depletion of norepinephrine from sympathetically innervated organs as a result of an acute and selective degeneration of the sympathetic adrenergic nerve (Tanzer and Thoenen, *Experientia* (Basel) 24: 155, 1968).

Four litters of rats will be studied beginning at birth, two litters will receive weekly subcutaneous injections of 6-OH dopamine (100 mg/kg). After reaching a body weight of 100 g, all four litters will be weaned and undergo unilateral nephrectomy. One litter of rats treated with 6-OH dopamine and one of untreated animals will be given deoxycorticosterone (DOCA) and 1% NaCl for 5 weeks. The other litter of rats treated with 6-OH dopamine and one of the untreated rats will serve as controls. All animals will be fed a regular laboratory diet.

8. Exposure to Smoke: Animals will be conditioned for at least one week prior to smoke exposure. Rats will be inserted into the animal cone holder and placed on the operating machine without cigarette, three times each day for 10 minutes. Suitably conditioned animals will enter the cone holders voluntarily.

Animals losing weight generally more than one gram per day during the conditioning period will be discarded, since these animals will not survive a chronic exposure. Following one week's exposure without smoke rats will be adopted with smoke to cigarette-concentration smoke for 8 minute exposure, 3 times a day.

The Walton Horizon Smoke Exposure Machine (developed under contract by the Council for Tobacco Research, U.S.A.) will be used. It has a capacity to expose 12 young rats to tobacco smoke (or simulated atmosphere) under conditions comparable to those of human smoke exposure.

Essentially smoke will be produced by "positive" puffing (blowing) meter air through a horizontally-held cigarette enclosed in a plastic dome during a timed two-second puff. The two-second puff interval is defined as the interval when the dome is in contact with the cigarette-holder plate. The average puff volume is defined as the average puff volume of smoke produced during the first eight puffs. The 35 ml is the average puff volume of smoke produced during the first eight puffs.

In the normal one-minute cycle of operation the two-second puff will be followed by a 15-sec. hold period, i.e., for a total exposure time of 17 sec. This will be followed by a 30 sec purge period to sweep out the smoke and a 13 sec rest period. The smoke will be pushed into a constant volume (384cc) smoke exposure chamber. Uniform mixing will be achieved with a mechanical mixer attached to one of the animal cone-holder plates.

1003541826

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 121-8885

Application for Research Grant
(Use extra pages as needed)

Date:
June 14, 1973

1. Principal Investigator (give title and degrees):

H. Fred Downey, Ph.D.
Assistant Professor of Physiology
Director, Cardiovascular Research
Cardiopulmonary Institute

2. Institution & address:

University of Texas Health Science Center at Dallas
5323 Harry Hines Blvd.
Dallas, Texas 75235

3. Department(s) where research will be done or collaboration provided:

Department of Physiology and
Cardiopulmonary Institute

4. Short title of study:

Effects of Tobacco Smoke and Nicotine on Coronary Collateral
Blood Flow

5. Proposed starting date: Soon, as Possible

6. Estimated time to complete: 2 years

7. Brief description of specific research aims.

- A. To determine the effects of
1. Tobacco smoke and
 2. Nicotine on coronary collateral blood flow following acute or chronic occlusion of a coronary artery.
- B. To determine the effects of these agents on the blood flow to other organs in the setting of acute and chronic coronary artery occlusion.

1003541837

13. Publications: Principal Investigator.

1. Downey, H. F., and E. S. Kirk. Coronary Lymph: Specific activities in interstitial fluid during uptake of ^{42}K . Am. J. Physiol. 215: 1177-1182, 1968.
2. Downey, J. M., H. F. Downey, and E. S. Kirk. Effect of myocardial strains on distribution of coronary blood flow in systole. Physiologist 13: 183, 1970.
3. Bashour, F. A., H. F. Downey, S. J. Kechejian, and R. Underwood. Effects of nitroglycerin on distribution of coronary blood flow following acute coronary occlusion. Clin. Res. 20: 767, Oct. 1972.
4. Bashour, F. A., A. Geumei, and H. F. Downey. Coronary vascular response to diphenylhydantoin. Clin. Res. 21: 80, 1973.
5. Downey, H. F., C. A. Bashour, C. S. Rutherford, and F. A. Bashour. Myocardial and total body extractions of radiorubidium. (Submitted for publication to the J. Appl. Physiol.)

Publications of Collaborator:

1. Parker, P. E., D. E. Dobbins, W. J. Weidner, F. J. Haddy, and G. J. Grega. Effects of hemorrhagic, endotoxin, and catecholamine shocks on canine gracilis muscle vasculature. Proc. Soc. Exp. Biol. Med. 138: 971, 1971.
2. DiSalvo, J., P. E. Parker, J. B. Scott, and F. J. Haddy. Carotid baroreceptor influence on coronary vascular resistance in the anesthetized dog. Am. J. Physiol. 221: 156, 1971.
3. Parker, P., J. Dabney, J. Scott, and F. Haddy. Cardiovascular effects evoked by selective stimulation of the carotid bodies with O_2 and CO_2 . Physiologist 14: 207, 1971.
4. Parker, P., J. Dabney, J. Scott, and F. Haddy. Vascular effects evoked in the kidney and intestine by selective stimulation of the carotid bodies with hypoxia and hypercapnia. Physiologist 15: 234, 1972.
5. Parker, P., I. Ehrhart, and J. Dabney. Vascular responses evoked in the heart and hindpaw by selective stimulation of the carotid bodies with hypoxia and hypercapnia. Fed. Proc. 32(3): 426, March, 1973.

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0.16; MgSO_4 , 7H₂O, 0.29; NaCl, 6.9; NaHCO_3 , 2.08 and glucose, 1.8. The temperature of the bath will be maintained at 37.5°C and the Krebs bicarbonate solution will be oxygenated with a mixture of 95 percent oxygen and 5 percent carbon dioxide. The strips will be subjected to an initial tension of 1 gram and will be kept in the organ bath for approximately 1 hour before drugs will be tested. Responses of the drugs will be measured isometrically with a force-displacement transducer and will be recorded on a polygraph as changes in tension in grams.

Chemical Methods

Animals will be killed by a blow on the head and decapitated. Various tissues will be rapidly removed, cleaned, frozen on dry ice and stored at 20°C prior to analysis.

1. Endogenous norepinephrine will be assayed by the method of Anton and Sayre (J.P.E.T. 133: 360, 1962). The method involves the selective absorption of catecholamines onto a constant amount of aluminum oxide, elution with a constant volume of perchloric acid and their measurement by the formation of fluorescent trihydroxyindole in the presence of potassium ferricyanide and alkaline ascorbare. To differentiate between epinephrine and norepinephrine, fluorescence is measured at 2 different pH's (pH 2-3 and pH 5-7). In the lower pH range, norepinephrine compared to epinephrine has a negligible fluorescence. Of the naturally occurring analogues of norepinephrine, only dopamine interferes, but this interference is reported to be relatively small. Samples will be run in duplicate and recovery rates of standard amount of epinephrine and norepinephrine are calculated for each analytical run. Recoveries up to at least 75% from biological materials have been reported.
2. ^3H -norepinephrine will be estimated by adding an aliquot of eluate (obtained after the alumina absorption of labelled amine as described above) in the counting solution (Instagel: Packard Instrument Co.) and the radioactivity will be determined in a Nuclear Chicago Scintillation counter.
3. ^3H -catechol deaminated metabolites will be assayed by the method of Kopin et al (J. Biol. Chem. 236: 2109, 1961).
4. ^3H -normetanephrine will be assayed by the method of Iversen et al (J.P.E.T., 150: 173, 1965).
5. ^3H -methylated deaminated metabolites will be estimated by the difference between the total radioactivity of the tissue extracts and the sum of other metabolites.

Enzyme Studies

Tissue will be removed, cleaned, weighed and homogenized in 2.0 ml of ice cold .25M sucrose. An aliquot (10 ul) of the homogenate will be

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METHODS

Isolated Atrial Preparation: The atria will be freed of ventricular muscle, connective tissue, fat and blood vessels; it will then be placed in a modified Tyrode's solution maintained at 34°C. A mixture of 95% oxygen and 5% carbon dioxide will be bubbled through the bathing fluid through a sintered glass plate at the bottom of the bath. The Tyrode's solution will have the following composition: NaCl, 0.9%; KCl, 0.04%; CaCl₂, 0.24%; NaHCO₃, 0.05%; glucose, 0.20%. The bicarbonate concentration employed will maintain the pH at approximately 7.4. The atria will be attached to a Grass force-displacement transducer; and isometric contractile force (resting tension of approximately 0.5 g) and rate of spontaneous contraction will be recorded by means of a Grass polygraph. The atria will be allowed to equilibrate at least 1 hour after being placed in the bath and will be washed repeatedly after each addition of the drug.

Left atrial strips driven electrically: The left atrium will be dissected from the heart and suspended in a (modified) Tyrode solution maintained at 34°C. It will be aerated with 95% and 5% CO₂. The lower end of the atrium will be tied to a plastic holder containing punctate electrodes. The upper end will be tied to a force-displacement transducer (Grass FT.03C) and contractions will be recorded on a Grass ink-writing oscillograph. Two atria (control and experimental) will be mounted in an organbath of 70 ml capacity. The atrium will be electrically driven via platinum electrodes, parallel to but not touching the tissue, with square-wave pulses of 5-msec duration, at frequency of 1/sec and above threshold voltage. The resting tension on the atria will be 1.0 g. The atria will be allowed to equilibrate for 1 hour after being placed in the bath and will be washed repeatedly after each addition of the drug.

Dose-response curve to sympathomimetic amines and other drugs. Cumulative dose-response curves to sympathomimetic amines, 5-hydroxy-tryptamine, and histamine will be determined by a stepwise increase of the total concentration. The concentration will be increased as soon as the response to the preceding dose reaches the maximal point (i.e., at intervals of 1 to 4 minutes).

To measure the sensitivity of atria to amines, the log concentration of the amine will be plotted against per cent of the maximum response. From each individual dose-response curve, a concentration which caused 50% of the maximum response will be calculated. The ratio ED-50 of the preparation, made from baboons pretreated with endotoxin, over ED-50 of control is a measure of sensitivity.

Aortic strips: Spirally cut thoracic aortic strips will be prepared by the method of Furchgott and Bhadrakom (J. Pharm. Exp. Ther. 108: 129, 1953). Each strip will be suspended in an isolated-organ bath (10 ml) containing a modified Krebs bicarbonate solution of the following composition (in gram per liter): KCl, 0.35; CaCl₂, H₂O, 0.37; KH₂PO₄,

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Cardiopulmonary Institute will provide the salaries of the principal and collaborating investigators. In addition, the Institute will provide adequate research laboratory, office, and animal facilities. Available in the laboratory for use in this investigation will be the following:

1. A six-channel physiological recorder with pressure, ECG, heart rate, and voltage couplers.
2. Micron electromagnetic blood flowmeters.
3. A triple-channel, 100 sample automatic gamma counter with teletype output and a PDP-8E computer for isotope separation analysis and general data processing.
4. Instrumentation laboratory pH and blood gas analyzer.
5. Respirators, perfusion and infusion pump, pressure transducers.
6. The Radiation Safety Section of the University of Texas Health Science Center at Dallas will provide facilities for storage and disposal of radioactive carcasses.

11. Additional facilities required:

None

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12. Biographical sketches of investigator(s) and other professional personnel (append).

13. Publications. (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Animals (conditioned for at least one week prior to smoke exposure) will be held in cone-shaped holder and will breathe the exposure chamber contents with their noses just inside the smoke chamber. They will be removed from the cone holder promptly after exposure to avoid water loss due to sweating and the additional stress of excessive confinement.

Cigarettes: Kentucky reference cigarettes (IRI) with different levels of nicotine will be used. They will be equilibrated for at least 24 hr at $76 (+ 2)^{\circ}\text{F}$ to $(+ 2)\%$ relative humidity atmosphere, by placing them unwrapped, with package opened into a dessicator (on wire mesh shelves) containing a 74% w/w glycerol-water solution in the bottom compartment. The cigarettes will be placed loosely into the chamber.

9. Pharmacological Studies: At various intervals of treatment the sensitivity of the cardiovascular system to selected drugs will be surveyed. These include norepinephrine, epinephrine, tyramine, tryptamine, isoproterenol, acetylcholine, atropine, mephentermine, methoxamine HCl, guanethidine, prostaglandin, propranolol. Chronotropic sensitivity as well as blood pressure responses will be determined.

Since the cardiac responses in the intact animal may be modified by reflex response an isolated atrial preparation will be used, thus eliminating their indirect action. Responses to sympathetic stimuli will be compared with those taken from untreated animals.

Reactivity of the vascular smooth muscle: Altered peripheral resistance of the vascular system is the characteristic of hypertension. We will, therefore, compare the reactivity in vitro of thoracic aortas from hypertensive animals with those from controls. The thoracic aorta will be used as an indicator of vascular reactivity because it can readily be prepared for the recording of pharmacologic responses, although the aorta exerts little, if any, effect on total peripheral resistance. Dose response curves to specific (norepinephrine, serotonin and histamine) and non-specific (potassium chloride) smooth muscle contractile substances will be determined. This will provide information as to whether there is an alteration in reactivity of the smooth muscle after endotoxin, and if this alteration is due to changes in the responsiveness of the muscle itself or due to specific modification of the receptors. If all the agonists are affected to about the same degree, then the mechanism responsible for this abnormality may be related to the intrinsic contractility of the muscle itself and not to a specific modification of receptors.

Catecholamines: In order to gain a better knowledge concerning uptake, storage, release and metabolism of catecholamines in the sympathetic nerve, neurotransmitter in the sympathetic nerve will be tagged with the radioactive catecholamine. Endogenous norepinephrine levels, rate of uptake of, binding and metabolism of H-norepinephrine in various tissues will be determined.

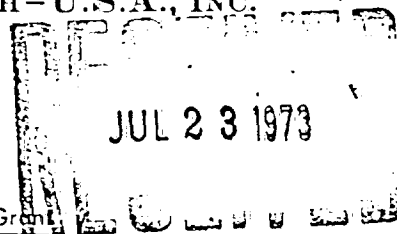
Turn-over rate of norepinephrine in tissue: Changes in turnover rate of tissue in catecholamines provides a more sensitive indication of sympathetic activity than do changes in the tissue concentrations of amine

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885



Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐

Second Renewal ☒

Date: July 15, 1973

1. Principal Investigator (give title and degrees):

Edwin R. Fisher, M.D., Director of Laboratories, Shadyside Hospital, 5230 Centre Avenue, Pittsburgh, Pa.; Professor of Pathology, University of Pittsburgh, Pittsburgh, Pa.

2. Institution & address:

Shadyside Hospital
5230 Centre Avenue
Pittsburgh, Pa. 15232

3. Department(s) where research will be done or collaboration provided:

Research will be done in Dept. of Pathology, Shadyside Hospital.
Collaborative help provided by Mark Wholey, M.D., Director, Division of Radiology, Shadyside Hospital.

4. Short title of study:

Effect of Tobacco Smoke and Nicotine on Structure and Function of Coronary Arteries and Plasma Lipids in Rabbits.

5. Proposed renewal date:

Anniversary Date - October 1, 1973

6. How results to date have changed earlier specific research aims:

None

7. How results to date have changed earlier working hypothesis:

None

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9. Experimental Design - Con't.

In other control experiments, placebo (saline) will be administered instead of nicotine. All other aspects of these experiments will be the same as in those where smoke or nicotine is administered. Data from these experiments will provide new information on any naturally occurring changes in coronary blood flow and its distribution after acute occlusion of a large coronary artery. This data will serve as a basis for evaluating alteration in collateral flow after treatment with either smoke or nicotine.

Inhalation of Tobacco Smoke. A lighted cigarette will be attached to one end of a tube connected to the air inflow port of the respirator.⁴ The portion of the inflow drawn through the cigarette will be adjusted so that the cigarette burns in approximately 5 min. Smoke from both regular and filter cigarettes will be studied.

After 5 min of exposure to smoke, differently labeled microspheres will be administered to map the distribution of coronary blood flow. Following this determination, exposure to smoke will be stopped. At various times in different experiments microspheres labelled with a third isotope will be administered to learn if the effects of smoke continue or are quickly reversed.

Nicotine infusion will be started after the base-line measurement of coronary flow distribution. The rate of infusion will initially be 0.20 $\mu\text{g/kg/min}$ for 5 min. Other infusion rates will be used as the investigation progresses to determine a dose-response curve. At 5 min the distribution of coronary flow will again be determined with the microsphere technique. After this determination, the infusion of nicotine will be stopped. Later a third determination of the distribution of coronary flow will be made.

Chronic coronary artery occlusion will be produced by surgically placing an ameroid constrictor around the LAD.^{8,9} These devices cause gradual, usually complete, occlusion of the artery over a period of weeks, allowing collateral vessels to develop. In most dogs these vessels supply sufficient coronary blood flow to prevent cardiac mortality and minimize myocardial necrosis. Even when infarcts occur, they are small, and adequate tissue supplied by collateral vessels is available for study.³

The dogs will be studied 6 weeks after implantation of the ameroid constrictors. As with the acute experiments, chloralose anesthesia will be used. Cannulae will be placed in the left ventricle for recording pressure and injection of the microspheres and in the femoral artery for collection of reference blood samples.⁵ A cannula will be introduced through a carotid artery into the aorta for recording arterial blood pressure. Rectal temperature, arterial blood gases and arterial pH will be monitored and kept within normal limits. ECG and heart rate will be recorded.

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ABSTRACT

A realistic daily pharmacologic dose of nicotine failed to quantitatively or qualitatively affect the atherosclerosis of aorta and extramural as well as intramural coronary arteries, visceral lesions, or serum lipids in normotensive or hypertensive rabbits with and without a dietary cholesterol supplement. No difference in the appearance of coronary angiograms could be appreciated in nicotine-treated rabbits with and without atherosclerosis. This technic did reveal less tortuous coronary arteries in all hypertensive rabbits which was reflected histologically by slightly greater luminal areas than in normotensive animals. Hypertension augmented the atherosclerotic process in the aorta and coronary arteries of cholesterol-fed rabbits. Nicotine failed to influence the induction or maintenance of renal hypertension. Although the clinical significance of these findings is uncertain, nevertheless they provoke the need for further inquiry concerning the role of nicotine, vis a vis cigarette smoking, and other determinants in the development of atherosclerotic heart disease in man.

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SIGNIFICANCE OF RESEARCH

Cigarette smoking has been implicated by epidemiological studies as one of the major hazards to health in the United States. Not only has it been associated with respiratory diseases and disorders, but it is also implicated in the development of cardiovascular diseases, particularly in cases of hypertension and coronary diseases. So far, no causal agent has been found to explain these clearly established statistical relationships. It seems obvious that knowledge of causal factors or mechanisms of these diseases will be of great importance in the understanding of the disease process and in determining appropriate treatment.

Does smoking accelerate the development and intensity of hypertension and if so, is it a simple provoking mechanism superimposed on existing susceptibility? The intention of this investigation is to examine systematically the influence of smoking on the cardiovascular system in normotensive and hypertensive rats, and to correlate changes in catecholamine pattern with the onset and degree of initial and subsequent hypertension. In this way, we hope to provide 1) evidence of causal relationship between cigarette smoking and hypertension and 2) understanding of the mechanism involved in those pathological processes.

It is the ambitious long-term aim of this project to work toward the achievement of such a breakthrough, or at least to affect significant advances in the field of hypertensive therapy by the continued exploration of pathogenesis of experimental hypertension. We are more than hopeful that our efforts will aid in the elucidation of the role of smoking in the development of cardiovascular diseases, particularly hypertension. A successful approach in this area would make it possible to develop preventive measures and help place therapy on a more logical basis. This would in turn decrease the mortality rate. It is also hoped that our findings when added to an enormous pool of data being accumulated on a national and international scale may provide a key to the riddle of essential human hypertension.

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The physiology and pharmacology of this vital circulation are not well understood, but there is reason to believe that tobacco smoke or nicotine might effect delivery of blood to ischemic myocardium. Collateral flow increases with arterial blood pressure.^{5,8,13} Conversely, pronounced dilation of coronary vessels in normal myocardium decreases coronary collateral blood flow.^{7,10} Although tobacco smoke and nicotine elevate arterial pressure, they also cause an autoregulatory dilation of the circulation in normal myocardium so that the net effect of these agents on coronary collateral flow must be experimentally determined. Furthermore, collateral coronary flow may be differently affected by tobacco smoke and nicotine if the development of collateral vessels has been stimulated by gradual, chronic occlusion of a major coronary artery.

Vasomotor responses to tobacco smoke or nicotine may alter the distribution of cardiac output. This distribution can be determined concomitantly with measurements of coronary collateral blood flow.

References have been made to the following publications:

1. Barger, L. M., D. Ehme, F. Gonlubol, A. Castellanos, A. Siegel, and R. J. Bing. Effect of cigarette smoking on coronary blood flow and myocardial metabolism. Circulation 15: 251-257, 1957.
2. Bellet, Samuel, N. T. DeGuzman, J. B. Kostis, L. Roman, and D. Fleischmann. The effect of inhalation of cigarette smoke on ventricular fibrillation threshold in normal dogs and dogs with acute myocardial infarction. Am. Heart J. 83: 67-76, 1972.
3. Bellet, S., J. W. West, O. F. Muller, and U. C. Manzoli. Effect of nicotine on the coronary blood flow and related circulatory parameters. Circ. Res. 10: 27-34, 1962.
4. Best, E. W. A Canadian study of smoking and health, Ottawa Department of National Health and Welfare, 1966, p. 137.
5. Corday, E., J. H. Williams, D. deVera, and H. Gold. Effect of systemic blood pressure and vasopressor drugs on coronary blood flow and the electrocardiogram. Am. J. Cardiol. 3: 626-637, 1959.
6. Corsini, G., P. S. Puri, P. V. M. Duran, and R. J. Bing. Effect of nicotine on capillary flow and vascular capacity on the heart in normal dogs and in animals with restricted coronary circulation. J. Pharmacol. Exp. Ther. 163: 353-361, 1968.
7. Downey, H. F., F. A. Bashour, and S. J. Kechejian. Dynamic effects of nitroglycerine on the distribution of coronary blood flow. Circulation 46: III-147, 1972.
8. Downey, H. F., and F. A. Bashour. Effect of perfusion pressure on transmural distribution of coronary collateral blood flow. Physiologist 15: 121, 1972.
9. Doyle, J. T., T. R. Dawber, W. B. Kannel, S. H. Kinch, and H. A. Kahn. The relationship of cigarette smoking to coronary heart disease. The second report of the combined experience of the Albany, N. Y., and Framingham, Mass., studies. J.A.M.A. 190: 886, 1964.

1003541839

Edwin R. Fisher, M.D.
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EFFECT OF TOBACCO SMOKE AND NICOTINE ON STRUCTURE AND FUNCTION OF CORONARY
ARTERIES AND PLASMA LIPIDS IN RABBITS

Since submission of the last Progress Report, the protocol concerned with the effect of nicotine administration on the structure and function of coronary arteries and plasma lipids in rabbits with and without various discriminants of atherosclerosis has been completed. A manuscript describing this investigation and the results obtained has already been accepted for publication in the Archives of Pathology. A copy of the pre-print is enclosed for perusal.

During the past year a "cigarette smoking machine" applicable for use in rabbits has been obtained to perform the protocol as originally outlined in regard to this form of nicotine consumption. Such studies are now in progress including smoking rabbits with and without induced renal hypertension and/or cholesterol atherosclerosis. Progress in this regard is relatively slow since the machine utilized accomodates only two animals per each exposure and each animal in all groups consumes 1 cigarette per day. Nevertheless, thus far the findings which are preliminary in this regard appear to parallel those observed following nicotine administration.

In addition, we have considered it worthwhile to obtain data on animals subjected to cigarette smoking for longer periods than originally outlined. Not only will this extended period of observation be more meaningful insofar as the cardiovascular effects of this form of nicotine administration but it will also allow us to obtain some meaningful histologic and ultrastructural

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INTRODUCTION AND SPECIFIC AIMS

There is enough evidence to suggest that cigarette smoking can contribute to the development of cardiovascular disease and particularly to death from coronary heart disease. Life expectancy among young men is reduced by an average of 7 to 8 years in heavy (over two packs a day) cigarette smokers and an average of 4 years in light (less than one-half pack a day) cigarette smokers. No substantial evidence has appeared to refute these forecasts.

While the exact mechanisms involved in the pathological effects of smoking are not known, the evidence suggests that cigarette smoking constitutes one of the major health hazards in the United States as well as in other parts of the world.

In normotensive persons, the intensity of sympathetic stimulation of the heart and blood vessels varies greatly with posture, activity, emotional state, physical conditioning and cardiovascular health. It influences venous capacitance, heart rate, myocardial contractility, as well as cardiac output and arteriolar resistance, the determinants of mean arterial pressure. Arterial pressure appears to be no less labile in hypertensive patients. This suggests that their sympathetic activity is also highly variable. This is further supported by the fact that the blood pressure of hypertensive men and animals is often lowered by the administration of drugs which alter the physiological disposition of NE, pointing toward substantial participation of this amine in the maintenance of high blood pressure. In addition, the increased vascular reactivity observed in some forms of human hypertension (Goldenberg et al., *Am. J. Med.* 5: 792, 1948) and experimental hypertension (Raab, *Am. J. Cardiol.* 4: 752, 1959) suggests that in these conditions there is either an impaired inactivation of amines or increased sensitivity of the effector cells.

Norepinephrine (NE) in the tissue innervated with sympathetic nerve endings is inactivated by at least three mechanisms: a) uptake and storage in nerve terminals, b) o-methylation by catechol-o-methyl transferase (COMT) and c) oxidative deamination by monoamine amine oxidase (MAO). Inactivation by uptake of NE is more important than inactivation by metabolism. In support of this is the observation that physiological effects of injected NE are rapidly terminated, even after both MAO and COMT are inhibited. Any drug or condition that prevents uptake or binding of NE would allow an increased amount of free catecholamine to remain in the vicinity of receptors, resulting in apparent supersensitivity to NE. Such a reduction in the myocardial accumulation of H^3 -NE in experimental hypertension was actually demonstrated by DeChamplain et al (*Life Science*, 5: 2283, 1966).

Biochemical evidence of altered sympathetic nerve function has been reported in essential hypertension (Brunjes, 5: *New Eng. J. Med.* 271: 120, 1964).

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Blocks of heart, lungs, aorta, small intestine, pancreas, spleen, kidneys, gonads, and thyroid were fixed in 10% neutral formalin; those of adrenal in both formalin and Orth's fluid and those of the extramural branches of the coronary arteries in gluteraldehyde. Paraffin sections were prepared in the usual manner and stained with hematoxylin and eosin. In addition, sections of coronary arteries, heart, and aorta were stained with thionin pH 4, 1:10,000 for estimation of metachromasia and orcein elastica and von Kossa calcium methods. Adrenals were stained by the ferric-ferricyanide chromaffin technic. Portions of coronary arteries fixed in gluteraldehyde were post fixed in 1% osmium tetroxide, dehydrated and imbedded in Maraglas. Ultrathin sections were examined by an EM 200 electron microscope.

The luminal area of extramural branches was computed from similarly magnified photographs of these structures by the formula $A = ab$. Comparisons of such measurements between groups were expressed as ratios.

Significance of differences between groups was estimated by the Student "t" test.

RESULTS

All animals exhibited a gain in body weight during the experimental period (Table I). This was least pronounced in hypertensive members.

Nicotine had no effect on body weight.

1003541866

12. Biographical sketches of investigator and other professional personnel:

H. Fred Downey, Ph.D.

BIRTHPLACE: **REDACTED**

EDUCATION:

University of Maryland 9/57-6/61 B.S. 6/61 Dairy Science

University of Maryland 9/61-1/64 M.S. 1/64 Dairy Science

University of Illinois 2/64-6/68 Ph.D. 6/68 Physiology and
Biophysics

POSITIONS HELD:

University of Maryland - Teaching Assistant, Dairy Science,
9/61 - 1/63

University of Illinois - Teaching Assistant, Physiology and
Biophysics, 9/65 - 9/66

University of Illinois - Assistant Professor, Veterinary
Physiology and Pharmacology, 7/68 - 1/72

University of Texas Southwestern Medical School - Assistant
Professor, Physiology, 2/72 - Present

ACADEMIC AND PROFESSIONAL HONORS:

B.S. With First Honors

Graduate Fellowship, 1961-1962, Alpha Zeta Honorary Fraternity

Research Fellowship, 1963, Oak Ridge Institute of Nuclear Studies

NIH Traineeship in Biophysics, 1964-1965

NIH Predoctoral Fellowship, 1966-1968

Invited Participant in Alfred Benzon Symposium II on
Capillary Permeability held in Copenhagen in 1969

PROFESSIONAL SOCIETIES AND RELATED ORGANIZATIONS:

REDACTED

REDACTED

1003541845

1003541885

#910 - RAMSEY

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Adenosine in Coronary Lymph	American Heart Assoc., Texas Affiliate	\$4,000	7/1/72-6/30/73
MI/Anti-Arrhythmic Drugs/Regional Coronary Blood Flow	American Heart Assoc., Texas Affiliate	\$7,500	7/1/73-6/30/74
Coronary Collateral Blood Flow	Cardiopulmonary Institute at Methodist Hospital of Dallas	\$2,500	1/1/73-12/31/73

PENDING OR PLANNED

Title of Project	Source (give grant numbers):	Amount	Inclusive Dates
Coronary Collateral Hemodynamics and Distribution	NIH		

1003541850

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name H. Fred Downey, Ph.D.Signature H. Fred Downey Date 6/15/73Telephone 214 - 946-8181, Ext. 378

Area Code

Number

Extension

Checks payable to

University of Texas Southwestern Medical School

Mailing address for checks

5323 Harry Hines Blvd.

Dallas, Texas 75235

Responsible officer of institution

Typed Name F. J. Bonte, M.D.Title DeanSignature F. J. Bonte Date _____Telephone 214 - 631-3220, Ext. 601

Area Code

Number

Extension

EXPERIMENTAL PROCEDURE

1. Preparation of Animals: Rats weighing about 60 to 70 gm will be used throughout this study. Animals will be placed in cages which will be kept under similar conditions of lighting and humidity in a room maintained at a temperature within the range of $21.0 \pm 0.5^{\circ}\text{C}$. Food and water will be supplied ad libitum. No more^x than 6 rats (unless otherwise required) will be housed in each cage, since it was observed that crowding of animals increased the tyrosine hydroxylase by 32%. All animals will be acclimatized to the new environment for a period of one week before they are subjected to any treatment.

2. Body Weight: Body weight will be recorded weekly.

3. Food and Water: Food and water intake will be measured daily and expressed per 100 gm of body weight.

4. Measurement of Systolic Blood Pressure: The systolic blood pressure will be measured weekly in unanesthetized animals using a pulse transducer applied to the tail. The blood pressure of rats anesthetized with pentobarbital (60 mg/kg) will be measured by cannulation of the left carotid artery through a statham strain gauge (Pd 23) transducer.

5. Sex Difference: Whether there is a sex difference in the effect of smoking experiments in the females will be compared with males. Some experiments will be performed on pregnant rats.

6. Representative Model for Essential Human Hypertension: Although a representative model for essential human hypertension is not yet available, the analysis of the factors determining the development of various forms of experimental hypertension may yield new insight in the pathogenesis of essential human hypertension and additionally lead to new therapeutic approaches.

Two models will be used. 1) DOCA-salt hypertensive rats, 2) genetically hypertensive rats (SHR).

1) Production of hypertension in rats: Rats weighing 80 to 90 g will be anesthetized with nembutal. Under aseptic conditions, the right kidney and adrenal gland will be removed. The rats will be made hypertensive by subcutaneous injections of a suspension of deoxycorticosterone pyruvate 10 mg per week and 1% NaCl solution to drink ad libitum for periods of 5-7 weeks. Both control and hypertensive animals will be fed a regular laboratory diet.

2) Spontaneous hypertensive rats: In 1963, Okamoto and Aoki (Jap. Cir. J. 271: 157, 1963) produced, by selective inbreeding a strain of Wistar rats with a 100% incidence of "spontaneous" hypertension. They (both male and female) will be used when they have reached an age of 3-14 weeks. They will be matched for age and body weight with a normotensive Wistar and a control obtained by backcrossing the spontaneously hypertensive rats with normotensive Wistar rats.

1003541825

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

July 24, 1973

Grant application No. 839R2

TO: The committee comprising Drs. Bing, Meier, and Sommers

SUBJECT: Edwin R. Fisher, M.D., Shadyside Hospital, Pittsburgh
Second Renewal Application No. 839R2
"Effect of Tobacco Smoke and Nicotine on Structure and
Function of Coronary Arteries and Plasma Lipids in
Rabbits"

History

Grant #839, effective October 1, 1971, had "priority in competition" recommended for two additional years.

Application #839R2, which would complete the initial three-year plan, requests \$24,978. (Some \$2,600. less than initially estimated).

Documents Submitted (attached)

1. Application dated July 15, 1973.
2. Progress Report No. 2.
3. "Influence of Nicotine on Experimental Atherosclerosis . . ." by Fisher et al., in press, Archives of Pathology.

Comment

The "cigarette smoking machine" referred to is a CTR small animal smoke exposure device.

FWN:gh

FWM
F.W.N.

1003541852

Epidemiologic studies have shown that cigarette smoking is associated with increased incidence and mortality rate from coronary artery disease.^{4,9,11,12,16} However, little is known about the direct effect of smoking or nicotine on coronary blood flow in ischemic myocardium. Such information is needed because of the large number of smokers who are suffering from regional myocardial ischemia.

Cigarette smoke and nicotine increase cardiac output, heart work, and coronary blood flow in normal experimental animals and man.^{1,3,14,19} Partial obstruction of the coronary circulation limits the coronary hyperemic response to the increased metabolic needs of myocardium stimulated by nicotine^{6,15,18} and under these conditions, coronary blood flow is distributed non-uniformly across the ischemic myocardium.¹⁵

No studies have reported the effects of smoking or nicotine on coronary collateral blood flow, although these agents have been shown to decrease ventricular fibrillation threshold in dogs with acute myocardial infarction.² However, if coronary arteries are obstructed gradually, collateral vessels develop which are sometimes able to meet minimal requirements of the myocardium in spite of complete obstruction of a major coronary artery.¹⁷

Continued on 2a

9. Details of experimental design and procedures (append extra pages as necessary)

Experimental Animals. All experiments will be conducted in adult, conditioned mongrel dogs of uniform size (18 to 23 kg). These animals will be examined by a veterinarian and certified free of respiratory diseases and heart worms. They will have been treated for intestinal parasites.

/ Acute Coronary Occlusion. To retain cardiovascular reflexes and normal cardiovascular dynamics, chloralose anesthesia will be used.⁶ The heart will be exposed through a left thoracotomy while respiration with room air is maintained with a Harvard ventilatory pump. Rectal temperature, arterial blood gases, and arterial pH will be monitored and kept within normal limits throughout the experiment. Routinely, aortic, left ventricular blood pressures, electrogram and heart rate will be recorded. Aortic and circumflex coronary artery blood flows will be measured with a dual-channel electromagnetic flowmeter. These flows will provide an index of cardiac output and flow to normal myocardium. The left anterior descending coronary artery (LAD) will be isolated about 2 cm from its origin and ligated according to the two-step procedure of Harris (partial occlusion for 5 min followed by total occlusion).⁷ Approximately 80% of the dogs will survive this insult.

Following coronary occlusion, the regional distribution of coronary blood flow will be measured with radioactive microspheres (8-10 μ diameter) administered via a cannula into the left ventricle, where they are well-mixed in the cardiac output.³ Microspheres reaching the region normally supplied by the LAD will reflect collateral flow, whereas those reaching tissue supplied by the left circumflex coronary artery will reflect normal (control) coronary flow.² Normally tissue supplied by the circumflex coronary artery and tissue supplied by the LAD are equally perfused.² Microspheres will be administered after occlusion of the LAD and before exposure to smoke or nicotine to provide base-line measurements of collateral flow in each heart. Subsequent injections of differently labeled microspheres will be made after exposure to tobacco smoke or nicotine to determine the distribution of coronary flow under experimental conditions.

Continued on 2c

1003541838

9. Experimental Design - Con't.

Coronary flow blood and its distribution will be determined with the microsphere technique. Differently labeled microspheres will be administered before, during, and after exposure to tobacco smoke or nicotine as described for the acute experiments. Two minutes after the final injection of microspheres the hearts will be stopped with saturated KCl iv, the chest opened and the heart excised for tissue sampling. The region of the Ameroid constrictor will be sectioned to determine the degree of narrowing of the coronary artery. Data from hearts with incomplete occlusions will be treated separately.

Measurement of Regional Coronary Blood Flow. Radioactive microspheres of 8-10 μ diameter (3-M Company) will be injected into the left ventricle before and after drug treatment.³ From there the microspheres are distributed to each tissue according to the fraction of the cardiac output it receives. Microspheres entering the coronary circulation are nearly 100% trapped in the myocardium and thus serve as an effective indicator of regional blood flow. By labeling the microspheres with three different isotopes, determinations of control (pre-treatment) and experimental blood flows (at two intervals post-treatment) can be made in the same heart.^{2,5} Since the extent of collateral development varies among dogs it is very helpful for each heart to serve as its own control.

Two minutes after the last injection of microspheres the heart will be excised and frozen for sampling. Tissue samples will be taken from the control, ischemic, and marginal myocardium. Ischemic tissue will be taken from the region normally supplied by the LAD and marginal tissue will be from the edge of the ischemic region. These samples will be divided transmurally into thirds so that the transmural distribution of flow can be determined. The samples will be weighed, and their radioactivities for each isotope determined by scintillation counting in a triple-channel gamma counter. Standard techniques for isotope separation will be utilized and accomplished with our PDP-8 computer.

Regional blood flow will be calculated by relating the radioactivity per gram of tissue with that of reference samples of arterial blood collected at a constant rate for 1 min after each injection of microspheres.⁵ This calculation uses the following formula:

$$MBF = \frac{\left(\frac{RBV}{Rcpm} \right) \times Mcpm}{CT \times \text{Tissue weight}}$$

MBF represents flow to a gram of tissue, RBV is the volume of the arterial blood sample collected as described above, Rcpm and Mcpm are the radioactivities of the reference blood sample and tissue

1003541842

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56. Fisher ER: Thyroidal influence on experimental cholesterol atherosclerosis. Am J Path 45: 21-39, 1964.
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1003541880

Abnormalities of tissue catecholamine metabolism in rabbits made hypertensive by complete denervation of the carotid sinuses and aortic arch (DeQuattro et al., *Cir. Res.* 24: 545, 1969) have been reported.

Rats made hypertensive with deoxycorticosterone (DOCA) and sodium have shown a hyperactivity of the sympathetic fibers and adrenal medulla. These rats seem to release more norepinephrine from the storage granules. This finding is reflected in lower endogenous norepinephrine levels, in the smaller proportion of ^3H -norepinephrine in the particulate as compared with the supernatant and in the increased excretion of norepinephrine and the deaminated and o-methylated metabolites in the kidney and urine (De Champlain et al., *Cir. Res.* 23: 479, 1968).

The spontaneously hypertensive rat (SHR) is another model of human essential hypertension (Okamoto and Aoki, *Jap. Cir. J.* 27: 282, 1963). It has been found that in the heart of SHR, norepinephrine turn-over rate was reduced in proportion to the rise in systolic blood pressure (Nakamura et al., *Naunyn-Schmiedebergs Arch. Pharmacol.* 271: 157, 1971).

Since there are alterations in the catecholamine patterns in the hypertensive patients and animals; since smoking is implicated in the development of cardiovascular diseases and particularly to death from coronary heart diseases and since nicotine, the principal alkaloid of tobacco produces its actions by release of catecholamines from its storage sites, it is therefore considered necessary to determine the effect of smoking on the synthesis and disposition of norepinephrine in the cardiovascular tissues in experimental hypertension.

In our proposed study, rats, both normotensive and hypertensive, will be exposed to tobacco smoke under conditions comparable to those of human smoke exposure. We will examine the following tissues: adrenal gland, superior cervical ganglia, heart, aorta, superior mesenteric artery, renal arteries, abdominal (inferior) vena cava and mesenteric vein. Changes in catecholamine pattern will be determined at various intervals following treatment (smoking) and following periods of withdrawal from cigarette smoking.

Thus the aim of the present proposals are:

To determine whether chronic smoking alters the catecholamine pattern in normotensive animals.

To determine how chronic smoking affects the altered pattern of catecholamines in experimental hypertensive rats.

To develop a more detailed understanding of the altered rate of synthesis and utilization of neurohormones in the cardiovascular tissues. We will examine animals at specific times following the start of smoking and (once the maximum changes in the catecholamine pattern have developed) during the subsequent period of cessation of smoking. An understanding of these factors is essential to attempt to define the mechanism involved in the synthesis and metabolism of these neurohormones.

1003541823

It is of interest that nicotine failed to affect the induction or maintenance of renal hypertension in the rabbit. Wenzel and Azmeh⁵⁷ noted similar results on the induction of renal hypertension in rats treated with nicotine, but a subsequent depressor effect after long-term treatment. Again the dose of nicotine was much greater than that utilized in our studies. It is well recognized that low doses of nicotine may be stimulating whereas the converse obtains with higher doses.

This study reaffirms the aggravating effect of hypertension on cholesterol-atherosclerosis. The coronary as well as other peripheral arteries in hypertensive rabbits not receiving the cholesterol diet and therefore lacking atherosclerosis, appeared less tortuous by angiography. This was reflected histologically by their slightly greater luminal area suggesting that uncomplicated hypertension may actually increase coronary blood flow.

It is appreciated that the results in the present study which fail to reveal any adverse effect of nicotine on the structural integrity of the cardiovascular system in rabbits with or without some other determinants of ASHD may not be applicable to the situation in man or other species. Nevertheless, they provoke the need for further study and scrutiny regarding the purported causal role of CS or nicotine in ASHD.

1003541873

a dose of nicotine which by our estimates appear equivalent to approximately 175 cigarettes a day in man or 5 fold that used in these present studies. It is of interest that they also observed an increase in serum phospholipids, which in our experience with cholesterol atherosclerosis in rabbits is attendant with a decreased severity of the vascular process.^{54,55} The failure of nicotine to influence aortic acid mucopolysaccharide content is in accord with our histochemical findings in the nicotine-treated animals. Increases in this moiety have been noted in this and other studies by us in situations in which the atherosclerotic process in rabbits is augmented.^{50,55,56} The studies of Lellouch et al³⁰ are difficult to evaluate since these investigators, utilizing a dose of nicotine equivalent to 525 cigarettes per day, found this agent to induce aortic subendothelial fibrosis which was unrelated to cholesterol-feeding, but mimicked that produced by adrenalin and was inhibited by monamine oxidase inhibitors. This lesion is unique for we have been unable to find any previous or subsequent accounts of a similar aortic change. Hass and associates³¹ similarly utilized an exceedingly high dose of nicotine as well as vitamin D in cholesterol-fed rabbits. They observed a pronounced medial effect on the aorta and other peripheral arteries including the coronaries as well as intimal change including thromboses in these latter vessels. It is quite apparent that one of the major sources of divergence of the results of these studies from our findings may reside largely in experimental design, particularly that concerned with the dose of the nicotine utilized which often appears to be in excess of that which may be regarded as realistic.

1003541872

Blood pressure was comparably ($P \geq .05$) but significantly ($P \leq .01$) elevated in hypertensive animals of all groups. Nicotine and/or hypercholesterolemia failed to affect the level of hypertension (Table I).

Total serum lipids, total cholesterol, triglycerides, phospholipids, and beta lipoproteins were significantly ($P \leq .01$) but comparably ($P \geq .05$) elevated in animals of all groups receiving the cholesterol diet. The administration of nicotine and/or presence of hypertension had no effect on these serum lipids in non-cholesterol-fed animal ($P \geq .05$). Total serum proteins appeared unaltered and similar in all groups (Table II).

No changes in serum calcium, phosphorus, bilirubin, or alkaline phosphatase were evident. LDH and SGOT although greater than that observed in man was in the normal range (LDH 175-350; SGOT 75-175) for control rabbits in all groups studied. Serum electrolytes were comparable in all groups. Urea N was slightly but not significantly elevated only in rabbits of Group III that were subjected to the induction of renal hypertension.

Weight of the heart was significantly ($P \leq .01$) increased in those groups of animals with hypertension, that of the adrenals only in those groups receiving the cholesterol diet ($P \leq .01$) (Table I).

Coronary angiography disclosed foci of atherosclerotic beading, and narrowing of one or more coronary arteries only in cholesterol-fed rabbits (Figs 1A & B, 2A & B). Such changes occurred at varying sites along the affected artery and were most frequent in the circumflex branch of the left coronary which appeared to be the predominant vessel in the rabbit. The

1003541867

CARDIOVASCULAR

1003541801

There have been many epidemiological studies which reveal a significant increase in the incidence of AsHD observed in man and are attributed to the stimulating effect of nicotine on the sympathetic nervous system and to catecholamine release.^{14,15,16}

The net effect of these actions has been interpreted to represent an adverse increased oxygen demand by the heart. It is noteworthy that doses of nicotine in dogs which are apparently devoid of systemic effects not only reproduce these changes but also result in increased coronary blood flow.¹⁷ This latter phenomenon appears to be confirmed by most recent studies concerning the effects of nicotine and/or CS on the cardiovascular system.¹⁸⁻²²

Retrospective pathological studies in man have for the most part disclosed varying degrees of increased aortic and coronary atherosclerosis in heavy smokers (generally more than 20 cigarettes per day) than in non-smokers.²³⁻²⁶

The age of men exhibiting sudden death due to a first episode of AsHD has been found to be 16 years less in heavy smokers than non-smokers and intermediate for ex-smokers and light smokers.²⁷ However, it should be indicated that most of these studies failed to consider other determinants such as serum lipids and hypertension which may influence the development of AsHD.

There have been surprisingly few experimental studies concerning the effect of CS or nicotine on the development of cardiovascular disease.²⁸⁻³⁵

The results have been conflicting and analysis of their significance is hampered by differences in species and techniques employed as well as varying doses of nicotine administered. Generally, the experimental designs have failed to consider other parameters which might influence or play a role in atherogenesis.

1003541862

REFERENCES (contd)

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41. Pozner H and Billimoria JD: Effect of smoking on blood clotting and lipid and lipoprotein levels. Lancet 760: 1318-1321, 1970.
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43. Gudbjarnason S: Effect of chronic nicotine administration on cholesterol metabolism of liver, serum, heart and brain. J Pharmacol and Exper Therapeut 161: 47-54, 1968.

1003541878

The purported significance of serum lipids in the pathogenesis of ASHD needs no elaboration. The effect of CS on this parameter has received a relatively modest amount of attention. Again, the results of investigations in this regard are inconsistent. Some have failed to note any immediate effects of CS in man upon serum cholesterol, phospholipids or triglycerides.^{36,37} Free fatty acids apparently increase after smoking although Frankl et al³⁸ believe this may represent an anxiety reaction to the tests being performed. Although cholesterol may be unaltered after smoking it is claimed by some,³⁹⁻⁴¹ but not others,⁴² that habitual smokers exhibit higher levels of cholesterol and beta lipoproteins than non-smokers. In animals, administration of nicotine has been noted to result in an immediate rise in serum triglycerides but not cholesterol, whereas, the converse appears to obtain in more chronic experiments.³⁷ A decreased rate of cholesterol synthesis as well as decrease of hepatic and myocardial cholesterol content has been observed in nicotine-treated dogs.⁴³ A few studies have been performed concerning the effect of CS on coagulation since alteration of this system may also represent another of the many factors concerned with atherogenesis. Generally, there is little or no effect on blood coagulation in smokers or after smoking⁴⁴ although increased platelet stickiness⁴⁵ and in vitro thrombus formation^{45,46} have been recorded.

The purpose of this present study was to investigate the pathologic effects of nicotine on cardiovascular and other tissues in rabbits as revealed by coronary angiography and appropriate histologic, histochemical and ultrastructural techniques. Such studies as well as those of serum lipids were performed in untreated rabbits and those subjected to such

1003541863

14. First year budget.

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

H. F. Downey, Ph.D.

40

P. E. Parker, Ph.D.

20

REDACTED

Technical

Arthur Williams, B.S.
Research Assistant

80

REDACTED

Animal Caretaker

25

Sub-Total for A

REDACTED

B. Consumable supplies (by major categories)

Dogs, conditioned 80 @ \$30

2,400

Radioactive microspheres

1,650

Maintenance, computer and Gamma counter

500

Miscellaneous Supplies (cannula, chemicals,
recorder and computer paper, counting
vials, anesthesia, occluders, etc.)

1,000

\$ 5,550

Sub-Total for B

C. Other expenses (itemize)

Travel to meeting of Federation of American
Societies for Experimental Biology or
American Heart Association

325

Sub-Total for C

\$ 325

Running Total of A + B + C

REDACTED

D. Permanent equipment (itemize)

Blood Flow Transducers 2 @ \$265

\$ 530

Sub-Total for D

\$ 530

E

1,925

E. Indirect costs (15% of A+B+C)

Total request

REDACTED

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2		5,900	300	None	2,055	\$ 15,755
Year 3	---	---	---	---	---	---

1003541848

TABLE II. SERUM LIPIDS, TOTAL PROTEIN (TP) IN
 (TP)/CHOLESTEROL-FED, HYPERTENSIVE AND NICOTINE-TREATED RABBITS

Group	Tot. Lipids (mg%)	Triglyc. (mg%)	Cholesterol (mg%)	P. Lipids (mg%)	Beta Lipoprot. (%)	Alpha Lipoprot. (%)	T.P. (Gm)
I. Cholesterol-fed	2402 \pm 500	386 \pm 102	1421 \pm 469	595 \pm 105	88	12	6.5 \pm .6
II. Nicotine	314 \pm 74	95 \pm 209	65 \pm 20	154 \pm 80	45	55	6.2 \pm .7
III. Hypertensive	320 \pm 63	112 \pm 35	57 \pm 76	151 \pm 76	55	45	6.6 \pm .7
IV. Hypert.+Nicotine	320 \pm 65	78 \pm 20	110 \pm 40	132 \pm 64	50	50	6.5 \pm .3
V. Hypert.+Cholesterol	2308 \pm 340	335 \pm 110	1200 \pm 320	773 \pm 120	85	15	7.0 \pm .8
VI. Cholesterol+Nicotine	224 \pm 610	296 \pm 54	1181 \pm 270	747 \pm 110	91	9	6.0 \pm .3
VII. Hypert.+Chol.+Nico.	2652 \pm 710	420 \pm 124	1347 \pm 420	885 \pm 98	88	12	6.1 \pm .4
Controls	370 \pm 82	102 \pm 30	71 \pm 24	197 \pm 66			

1003541882

LEGENDS (contd)

rabbit. H & E X 40.

Fig. 7. Higher magnification of focus of myocardial necrosis depicted in Fig. 7. H & E X 240.

1003541884

TABLE I. BODY WEIGHT, BLOOD PRESSURE AND ORGAN WEIGHTS OF CHOLESTEROL-FED, HYPERTENSIVE, NICOTINE-TREATED RABBITS

Group	Change body wt. (Kg)	Blood pressure mmHg		Heart	Organ weights (Gm)		Adrenals
		Init.	Final		Liver		
I. Cholesterol-fed	+1.0	96 \pm 12	104 \pm 10	5.6 \pm .8	95 \pm 30		1.070 \pm .240
II. Nicotine	+1.2	105 \pm 8	108 \pm 11	6.5 \pm .9	104 \pm 20		.490 \pm .170
III. Hypertensive	+.7	105 \pm 8	145 \pm 12	8.8 \pm .4	99 \pm 30		.490 \pm .240
IV. Hypert.+Nicotine	+.8	103 \pm 7	140 \pm 7	8.6 \pm .8	90 \pm 30		.500 \pm .130
V. Hypert. + Cholesterol	+.8	100 \pm 10	148 \pm 14	8.5 \pm .6	100 \pm 32		.988 \pm .120
VI. Cholesterol+Nicotine	+.9	110 \pm 7	110 \pm 11	6.0 \pm .7	105 \pm 25		.990 \pm .290
VII. Hypert.+Chol.+Nico.	+.7	105 \pm 10	138 \pm 8	8.8 \pm .7	108 \pm 28		1.200 \pm .320

1003541881

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Ultrastructural Studies in Human and Experimental Pathology	Shadyside Hospital Laboratory Research Fund	10,000/ annum	yearly

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

1003541856

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Edwin R. Fisher, M.D.Signature *Edwin R. Fisher* Date 7/19/73Telephone 412 622 2315
Area Code Number Extension

Responsible officer of institution

Typed Name David HaldemanTitle Director of Fiscal AffairsSignature *David Haldeman* Date 7/19/73Telephone 412 622 2036
Area Code Number Extension

Checks payable to

David Haldeman
Director of Fiscal Affairs

Mailing address for checks:

Shadyside Hospital
5230 Centre Avenue
Pittsburgh, Pa. 15232

Some of the points worth considering in subsequent years would be the role of erythrocytic 2,3-diphosphoglycerate concentrations in response to carbon monoxide exposures as well as evaluating more specific involvement of erythropoietin and its mechanisms in regulating the red cell production, Hb synthesis, etc., in carbon monoxide exposures. Erythropoietic suppression, RBC destruction, and Hb catabolic rates in the liver would offer still other related avenues of investigation.

1003541892

which might remain constant or even decline in spite of an increased rate of synthesis. Therefore, turn-over rate of norepinephrine in cardiovascular tissue will be determined according to steady-state kinetics (Brodie et al, J. Pharmacol. & Exp. Ther. 154: 493, 1966).

Tyrosine hydroxylase activity: The catecholamines, norepinephrine and epinephrine, are continuously being synthesized, released and metabolized. However, tissue catecholamines remain at a steady level characteristic of each organ. It appears that there is a dynamic balance between the rate of synthesis of norepinephrine and its disappearance.

The procedures which increase sympathetic nervous activity, such as exposure to cold or heat, exercise, α -receptor blockade, thyroidectomy and electrical stimulation of sympathetic nerves, produce an increase in the synthesis of norepinephrine as a result of increased tyrosine hydroxylase activity. However, this effect occurs without an increase in the amount of enzyme. The increase in enzyme activity is due to release of tyrosine hydroxylase from the end-product inhibition.

It is recently reported that in rats rendered hypertensive by carotid sinus denervation, the content of tyrosine hydroxylase in the heart was significantly greater than that observed in hearts of control animals (De Quattro, et al, Fed. Proc. 27: 240, 1968). Conceivably, chronically increased sympathetic nervous activity may lead to increased synthesis of tyrosine hydroxylase and an increased content of this enzyme in adrenergic nervous tissue. This phenomenon may be highly significant in the pathogenesis of disease states where an increased sympathetic nervous activity is a significant component.

The chronic increase in the sympathoadrenal activity induced by exposure to severe stresses has been shown to elevate adrenal tyrosine hydroxylase levels (Mueller et al, J.P.E.T. 169: 74-79, 1969).

Since smoking results in an increased sympathoadrenal activity, it will be interesting to measure tyrosine hydroxylase activity in various tissues.

Phenylethanol-N-Methyl Transferase (PNMT) in adrenal glands: PNMT is another enzyme which converts norepinephrine to epinephrine in the adrenal gland. The activity of PNMT increases in response to various conditions or treatments which increase the tyrosine hydroxylase activity. These include insulin-induced hypoglycemia (Patrick and Kirshner, Mol. Pharmacol., 7: 87, 1971), administration of reserpine (Bhagat et al, Br. J. Pharmac. 43: 819, 1971) or 6 hydroxydopamine (Thoenen et al, Biochem. Pharmacol. 19: 669, 1970) immobilization stress (Kvetnansky et al, Endocrinology 87: 744, 1970) and stress by prolonged isolation or by repeated exposure to cold. In all these conditions activation of the sympathoadrenal system and enhanced secretion of catecholamines are the common denominator. Since smoking and administration of nicotine results in an increased sympathoadrenal activity, it will be interesting to measure PNMT activity of the adrenal gland in the rat. We have already evidence that PNMT activity in adrenal gland is increased after chronic treatment with nicotine (Bhagat and Rana, Brit. J. Pharmac. 43: 250, 1971).

1003541828

*

INFLUENCE OF NICOTINE ON EXPERIMENTAL ATHEROSCLEROSIS AND ITS DETERMINANTS

Edwin R. Fisher, M.D.; R. Rothstein, M.S., Mark H. Wholey, M.D. and R. Nelson, M.S.

*Supported by Grant #839R1 from the Council for Tobacco Research-U.S.A.

From the Departments of Pathology and Radiology Shadyside Hospital and
University of Pittsburgh, Pittsburgh, Pennsylvania.

Address for reprints: Edwin R. Fisher, M. D., Institute of Pathology,
Shadyside Hospital, 5230 Centre Avenue, Pittsburgh, Pennsylvania .

1003541859

#814R2 - RYAN

1003541909

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Two modern, air conditioned research laboratory areas in the Biology Department are under the direction of the proposed Principal Investigator for studies in respiratory physiology, hematology, and environmental physiology. The main laboratory, Sherman 125, is equipped with instrumentation for blood gas analyses, pulmonary performance testing, cardiac and circulatory evaluations, and standard hematology. This laboratory also has extensive files of reprints related to CO toxicology.

The second laboratory area, Sherman 229, is where exposure chambers for both humans and animals are housed. The human environmental chamber is a huge, walk-in facility comfortably capable of handling ten individuals at a time. Temperature and relative humidity within are precisely controlled. For the most part, very little in the way of additional equipment is needed for the proposed study. An additional hemophotometer for Hb evaluations could expedite processing eight daily determinations, i. e., two technicians could share the eight determinations with two instruments. Also, an additional centrifuge (handling 15 ml tubes, \$120) could be used in working with plasma volume determinations.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

See pages 8 and 9.

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See pages 12 and 13.

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#889RL - REGAN

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9. Experimental Design - Con't.

sample respectively, and CT is the collection time of the arterial sample (1 min). Two reference samples will be collected simultaneously through two small cannulae of different lengths inserted through the femoral artery into the abdominal aorta. Similar radioactivities in these samples will verify that the microspheres were well mixed in arterial blood.

This procedure for measuring regional coronary flow is basically the same technique used by Hoffman's group.⁵ We are aware of the need to limit the number of microspheres injected so as not to alter systemic and coronary hemodynamics. Also, sufficiently large samples will be counted to minimize statistical errors in the counting procedures for determining radioactivities. In the process of determining collateral myocardial flow, tissue samples from other organs will be obtained and their respective flow determined.

We are presently engaged in an investigation of the effects of anti-anginal and anti-arrhythmic agents on coronary collateral flow using the same approach outlined in this proposal.¹ This experience will permit us to proceed most efficiently with the proposed investigation. Also, we are experienced in preparing dogs with chronic coronary occlusions.

References have been made to the following publications:

1. Bashour, F. A., H. F. Downey, S. Kechejian, and R. Underwood. Effects of nitroglycerine on distribution of coronary blood flow following acute coronary occlusion. Clin. Res. 20: 767, 1972.
2. Becker, L. C., N. J. Fortuin, and B. Pitt. Effect of ischemia and antianginal drugs on the distribution of radioactive microspheres in the canine left ventricle. Circ. Res. 28: 263-269, 1971.
3. Becker, Lewis C., and Bertram Pitt. Collateral blood flow in conscious dogs with chronic coronary artery occlusion. Am. J. Physiol. 221: 1507-1510, 1971.
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1003541843

Public Health Service (NAPCA), Research Related to Carbon Monoxide Toxicology, December 1969-April 1971, \$12,682;

National Science Foundation, Research Related to Carbon Monoxide Toxicology, July 1971-1972, \$3,000; National Science Foundation, Research Related to Carbon Monoxide Toxicology, November 1972-1973, \$2,825

Publications: The above studies have resulted in 10 publications (one in press), seven of which are cited below.

"Carboxyhemoglobinemia in Parking Garage Employees," J. M. Ramsey, Arch. Environ. Health, Vol. 15, November 1967.

"Potassium Pallado Sulfite Detection of Carbon Monoxide in Exhaled Air as an Estimate of Carboxyhemoglobin," J. M. Ramsey, Amer. Indust. Hyg. Assoc. J., Vol. 28, December 1967.

*"The Immediate Haematological Response in the Rat to Experimental Exposures of Carbon Monoxide," J. M. Ramsey, Jour. Physiol., 202:297-304, 1969.

*"The Time Course of Hematological Response to Experimental Exposures of Carbon Monoxide," J. M. Ramsey, Arch. Environ. Health, 18:323-329, March 1969.

*"Oxygen Reduction and Reaction Time in Hypoxic and Normal Drivers," J. M. Ramsey, Arch. Environ. Health, 20:597-601, May 1970.

*"Carbon Monoxide, Tissue Hypoxia, and Sensory, Psychomotor Response in Hypoxaemic Subjects," J. M. Ramsey, Clinical Science, 42:619-625, May 1972.

"The Effects of Single Exposures of Carbon Monoxide on Sensory and Psychomotor Response," J. M. Ramsey, Amer. Indust. Hyg. Assoc. J., 1973 (in press).

*Reprint included with proposal.

1003541895

similar size (600-1200 u luminal diameter) (Fig 4) and the intramural branches of the coronary arteries with luminal diameter of 40-660 u. (Fig 5). In the former, the atheromatous lesions resembled those of the aorta with distinct foam cells, varying amounts of collagen and amorphous lipid deposits, whereas those of intramural branches consisted almost exclusively of large, irregularly shaped acellular collections of optically clear lipid with indistinct cells borders and only occasional nuclei. This lesion often appeared to obliterate the lumen. The media of these involved vessels was markedly thinned. The ratio of the luminal area of extramural coronary arteries of hypertensive rabbits to that of normotensive members was 1.5-1.7 whereas that of other groups more closely approximated 1.

The ultrastructural features of cholesterol atheroma in coronary arteries were comparable to those described previously in aortas of cholesterol-fed rabbits by others.⁵¹⁻⁵³ Nicotine and/or hypertension failed to influence these changes in cholesterol-fed animals or the normal appearance of these vessels in those receiving the non-cholesterol diet.

Sections of heart from approximately $\frac{1}{4}$ of the rabbits from cholesterol-fed groups exhibited miliary infarcts (Figs 6 & 7) or foci of subendocardial necrosis in the myocardium of the left ventricle. In addition, interstitial infiltrates of foam cells with or without other inflammatory infiltrate were also observed in $\frac{1}{4}$ of cholesterol-fed animals. These appeared to be most pronounced in rabbits with hypertension and not related to nicotine treatment or levels of serum lipids.

1003541869

Publications Continued:

Smith, U. and Ryan, J.W.: Electron microscopy of endothelial cells collected on cellulose acetate paper. *Tissue & Cell*, 5:333, 1973.

Smith, U. and Ryan, J.W.: Electron microscopy of endothelial and epithelial components of the lungs: Correlations of structure and function. *Fed. Proc.*, in press.

1003541913

In respect to hematological effects of CO exposures with man, the picture is even more discrepant. In substantial and prolonged exposures to one human subject, Killick obtained no changes in RBC count, reticulocyte proportion, or in blood volume. Using a limited number of rather high (12 to 30% COHb) intermittent exposures, Ramsey showed some elevation in mean hematocrit and Hb content of eight subjects. However, the elevations were not statistically significant in every exposure. Siggard-Andersen et al., used eight days of exposures (five times per day) resulting in 11% COHb and obtained no significant change in plasma volumes. Kjeldsen and Damgaard exposed eight subjects intermittently for eight to ten days (13% COHb). They obtained a moderate increase in reticulocytes but nonsignificant changes in hematocrit. The same subjects exposed later to 3,500 meters altitude showed a gradual increase in hematocrit. Finne claims that hypoxia, anemic or hypoxic, will result in increased production of erythropoietin within 12 hours. Some writers (Dinman) (Beard) have stated that long-term CO exposures may produce increased hematocrits and Hb, but that available data are inadequate to draw conclusions. Finally, Eisen and Hammond, working with habitual smokers who were asked to refrain from smoking for various periods of time, claim that hematocrits, RBC counts, and Hb were found to be higher during periods of smoking than during abstinence.

It is obvious that it simply isn't clear whether or not chronic, low level, intermittent exposures of CO can result in significant polycythemia or significant increases in blood viscosity. The situation with CO is not nearly so clear as is the case with hypobaric O₂. In anemic hypoxia (which is what CO exposures amount to), the arterial PO₂ is not significantly reduced. If indeed the arterial PO₂ is the primary trigger in erythropoiesis, then perhaps low level CO may not be capable of producing polycythemia.

1003541889

BUDGET JUSTIFICATION

*Una Ryan (formerly Una Smith, please see letter to Dr. Hockett, dated July 30, 1973). The major portion of Dr. Una Ryan's salary is paid by the American Heart Association through her tenure of an Established Investigatorship. The salary requested here is calculated as 25% of the allowed supplement.

**James W. Ryan and Peter C. Moller. Salary funds are requested in proportion to per cent times to be spent on this research project. Dr. P.C. Moller replaced Dr. Doris Chang, who has returned to Taiwan. Dr. Moller's biographical sketch is attached.

Fringe benefits, including social security, health insurance, life insurance and unemployment, are calculated as 10% of salaries.

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Blood pressure was estimated indirectly at biweekly intervals by the ear capsule technic of Grant and Rothschild.⁴⁹

All biochemical reactions were performed on aortic blood obtained at the time of sacrifice after an overnight fast. Total lipids were determined by the phospho-vanillin reaction; triglycerides by the automated colorimetric periodate reaction; total cholesterol by the method of Lieberman and Burchard and phospholipids by differentiation. Beta and alpha lipoproteins were calculated as percent of lipoproteins from cellulose acetate electrophoretograms stained with oil red O and total proteins by the biuret reaction. Serum calcium, phosphorus, urea N, bilirubin, alkaline phosphatase, LDH, SGOT and electrolytes by the methods utilized with the "Autoanalyzer".

Coronary angiography was performed by catheterization of the left femoral artery. The catheter was positioned either selectively in the left coronary orifice or at the root of the aorta at the level of the aortic cusps by television fluoroscopy. Injections of methyl glucamine diatrizoate (Renograffin 76) was accomplished by flow rate control at 6 ml/sec for a total of 8 ml. In instances of selective angiography 1 ml was delivered by manual controlled flow. Films were exposed on a Franklin roll film changer at a rate of 4/sec for two seconds. At least 5 animals in each group had successful coronary angiograms performed just prior to sacrifice.

At the time of sacrifice the heart, liver, adrenals and spleen were cleaned and weighed. The degree of aortic atherosclerosis was determined arbitrarily by computing the average grades of atherosclerosis of both the thoracic and abdominal portions as described previously.⁵⁰

1003541865

Figure Caption

Electron micrograph showing lamellar bodies giving the appearance of unravelling to yield tubular myelin after expulsion into the air space.

X 32,500

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degree of change appeared to be unaffected by the administration of nicotine, but was more pronounced in the hypertensive, cholesterol-fed animals. Angiographically, coronaries of otherwise untreated hypertensive rabbits were less tortuous than those of other groups.

Hypertensive, cholesterol-fed rabbits exhibited more extensive aortic atherosclerosis than normotensive cholesterol-fed animals (Fig 3). Nicotine administration failed to influence the severity of aortic atherosclerosis. No atherosclerosis or other vascular changes were observed in nicotine-treated or hypertensive animals not receiving the cholesterol diet.

The histopathological appearances, degree of elastica alteration and intimal calcium deposition of the lesions of the aorta, coronary and other arteries were qualitatively similar in all cholesterol-fed rabbits regardless of presence or absence of hypertension or administration of nicotine and have been recounted in detail previously.⁵⁰ Metachromasia appeared increased in aortas from all hypertensive rabbits whether or not they received the cholesterol diet. In these instances the metachromatic material was evident throughout the entire medial coat as well as in the intimal lesions of cholesterol-fed members. A slight increase in metachromasia was apparent in the media of the extramural branches of the coronary arteries of all hypertensive animals only, but this change was less consistent in other systemic arteries of these animals. No effect of nicotine treatment on the degree of metachromasia was appreciated.

A qualitative difference in the type of intimal atherosclerosis existed between the lesions of extramural coronary and distributing arteries of

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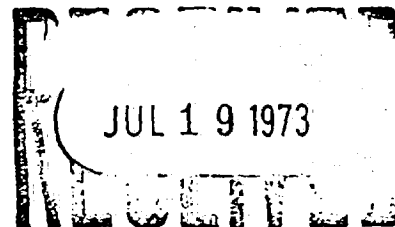
Drs. Bing
Gardner
Sommers

CARDIOVASCULAR

#889R1

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885



Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒

Second Renewal ☐

Date: 07/10/73

1. Principal Investigator (give title and degrees): Timothy J. Regan, M.D.
Professor of Medicine
Director, Division of Cardiovascular Disease
2. Institution & address: College of Medicine & Dentistry of New Jersey-New Jersey Medical School
100 Bergen Street
Newark, New Jersey 07103
3. Department(s) where research will be done or collaboration provided: Division of Cardiovascular Disease
Department of Medicine
New Jersey Medical School
4. Short title of study: Variables affecting the cardiovascular responses to chronic smoking.
5. Proposed renewal date: January 1, 1974 to December 31, 1974
6. How results to date have changed earlier specific research aims:

In these chronic studies the animals have not been in the program sufficiently long to warrant a change in our specific research aims.

7. How results to date have changed earlier working hypothesis:

Refer to item # 6.

1003541902

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

July 19, 1973

Grant application No. 889R1

TO: The committee comprising Drs. Bing, Gardner and Sommers

SUBJECT: Timothy J. Regan, M.D., College of Medicine and Dentistry of
New Jersey, Newark
First Renewal Application No. 889R1
"Variables affecting the cardiovascular responses to chronic
smoking"

History

Grant #889, for the calendar year 1973, was awarded in the amount requested, \$44,776. Also voted was "priority in competition" for two additional years.

Application #889R1 requests \$45,500., exactly the amount originally estimated for this year.

Documents Submitted (attached)

1. Application dated 07/10/73.
2. Progress Report No. 1, 01/01/73 - 06/30/73.

Comment

Of note are the studies of human beings (summarized on page 1-a of the Progress Report) not called for in the original application.

FWN:gh

ENCLOSURES

F.W.N.
F.W.N.

1003541901

BIBLIOGRAPHY

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Cavusoglu, H. and A. Kayserilioglu. "The Effects of General Hypoxia and Renal Ischemia on Erythropoietin Production," Arch. Intern. de Physiol. et de Biochim., 77:260, 1969.

Dinman, B. D. "Effects of Long-Term Exposure to Carbon Monoxide," Nat. Acad. of Sciences, Washington, D. C., 1969, p. 25.

Eisen, M. E. and E. C. Hammond. "The Effect of Smoking on Packed Cell Volume, Red Blood Cell Counts, Haemoglobin, and Platelet Counts," Canad. Med. Assoc. J., 75:520, September 1956.

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Killick, E. M. "The Nature of the Acclimatization Occurring During Repeated Exposure of the Human Subject to Atmospheres Containing Low Concentrations of Carbon Monoxide," J. Physiol., 107:27, 1948.

Kjeldsen, K. and F. Damgaard. "Influence of Prolonged Carbon Monoxide Exposure and High Altitude on the Composition of Blood and Urine in Man," Scand. J. Clin. and Lab. Invest., 22:20, 1968.

1003541838

Appendix II.

Item 10. Outline of Experimental Protocols for Ensuing Year.

1. Arterial blood pressure and heart rate in the squirrel monkey during behavioral procedures following the administration of nicotine.

Blood pressures and heart rates of squirrel monkeys will be measured using an oscillometric technique before and after daily sessions under operant conditioning procedures. As soon as consistent behavioral and cardiovascular responses to the operant conditioning schedules have been established, nicotine tartrate (0.01 to 1.0 mg/kg, i.m.), chlordiazepoxide (1.0 to 30.0 mg/kg, i.m.), d-amphetamine (0.01 to 1.0 mg/kg, i.m.) or saline (1.0 ml, i.m.) will be administered before the start of each daily session. Additional experiments will also be performed in which these agents are administered chronically over long periods of time.

2. Effects of nicotine on hypercholesterolemia and aortic atherosclerosis in the squirrel monkey during administration of an atherogenic diet.

Young adult squirrel monkeys will be trained initially to eat a semi-purified diet of normal composition and, later, a diet containing moderate amounts of saturated fats (8 g %) and cholesterol (0.1 to 0.2 g %). Venous blood will be drawn repeatedly for measurements of serum cholesterol levels. As soon as intake of food and water, body weight, and serum cholesterol values of all subjects have stabilized during the administration of an atherogenic diet, nicotine tartrate (0.01 to 1.0 mg/kg·day, p.o.) will be administered chronically. Some animals will be continued on each regimen for 16 weeks and then examined using standard techniques for investigating gross and microscopic vascular pathologic anatomy. Some animals will be treated with propranolol (0.1 to 10 mg/kg·day, p.o.) or guanethidine (0.01 to 1.0 mg/kg·day, p.o.) in addition to cholesterol and saturated fats in the diet and nicotine tartrate in the drinking water.

3. Arterial blood pressure and oxygen consumption in the squirrel monkey at high and low ambient temperatures.

Blood pressures, heart rates, and oxygen consumptions of adult squirrel monkeys will be measured before and after daily sessions under operant conditioning procedures at high and low ambient temperatures. As soon as consistent behavioral, metabolic, and cardiovascular responses have been established, nicotine tartrate (0.01 to 1.0 mg/kg, i.m.) or saline (1.0 ml, i.m.) will be administered before the start of each daily session. Some animals will be subjected to surgical denervation of the carotid sinuses and the aortic arch to enhance the cardiovascular and metabolic responses to behavioral procedures at low ambient temperatures. The role of the sympathetic nervous system in mediating the responses to behavioral procedures at low ambient temperatures will be studied using intravenous infusions of alpha-adrenergic agonists such as phenylephrine and vasodilator substances such as glyceryltrinitrate, diazoxide, and phentolamine.

4. Effects of carbon monoxide on hypercholesterolemia and aortic atherosclerosis in the squirrel monkey during administration of an atherogenic diet.

In these studies, an experimental protocol similar to that described in section 2, Item 10 above will be followed. As soon as intakes of food and water, body weights, and serum cholesterol values have stabilized during the administration of an atherogenic diet, carbon monoxide will be added to the inspired air in concentrations of 50 to 250 p.p.m. Effects of carbon monoxide

1003541809

REFERENCES

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8. Coffman JD: Effect of propranolol on blood pressure and skin flow during cigarette smoking. J Clin Pharmacol 9: 39-44, 1969.

1003541874

Of importance to our original goals, we further found that the frequency of the points of fusion is increased in glands perfused with angiotensin II and is vastly decreased when calcium is omitted from the perfusion system (Rubin, 1970). So far as we are aware, our studies are the first to demonstrate a discrete ultrastructural effect of angiotensin II.

Significance

Although gas exchange between blood and air is undoubtedly the fundamental function of the alveolar capillary unit, there is a growing body of direct and indirect evidence which indicates that the endothelial cells and type II alveolar cells carry out specialized metabolic functions of possible importance not only to the performance of the lungs themselves but also to activities of distant organs and glands. Our previous studies of these "non-ventilatory" functions suggest that it is feasible to relate the functions to fine structure by using advanced techniques of tissue preparation and high magnification electron microscopy.

1003541926

No distinct qualitative or quantitative differences in lipid deposits in other viscera were apparent in any group studied or in the numbers of arteries in them involved with atheromatous plaques in cholesterol-fed rabbits. No differences in ferric-ferricyanide reactive chromaffin tissue of adrenal medullas was apparent in any group studied.

Although the numbers of each sex were small in each group there did not appear to be any qualitative or quantitative differences in the atherosclerosis, angiographic, or biochemical findings in males and females.

No increase in incidence of spontaneous medial lesions was encountered in any group.

DISCUSSION

The results of these studies fail to disclose any significant influence of nicotine on the severity, histopathologic, untrastructural, histochemical or angiographic features of aortas and coronary arteries or serum lipids of otherwise untreated rabbits or those subjected to such determinants of atherosclerosis as hypercholesterolemia and/or hypertension. Similarly, the incidence and/or severity of rare foci of myocardial necrosis was unaffected by nicotine administration. These myocardial changes have not been observed with any degree of frequency in cholesterol-fed rabbits not receiving other thrombogenic factors, but was relatively conspicuous in this study. On the other hand, overt myocardial infarction was not observed and might be explained by the predominant and often exclusive involvement of the circumflex branch of the coronary system, a situation unlike that observed in man.

1003541870

The failure of nicotine to augment the atherosclerotic process even in cholesterol-fed rabbits with hypertension is in agreement with results of previous studies in the rat ³² and the dog ³⁷ which fail to ascribe any pathologic changes in the cardiovascular system to nicotine. It should be noted that these species are quite resistant to atherosclerosis even in the presence of hypercholesterolemia.

In the rabbit, Wenzel et al ^{34,35} using graded doses of nicotine in drinking water failed to discern any effect of this agent on aortic atherosclerosis. However, they did record "thickening and fibrosis in small branches of coronaries" following nicotine treatment although details in this regard were not presented or depicted. Further, occlusive coronary changes were encountered in nicotine-treated, cholesterol-fed rabbits accompanied by myocardial necrosis. Since this was not apparent in nicotine treated members not fed cholesterol they proposed that these changes were due to some interaction between the two. Not only do these authors disregard the possibility that these changes may be due to cholesterolemia per se, as is shown in this study, but also it is evident that the references to atherosclerotic involvement is concerned with intramural branches of the coronary arteries only. In addition, these investigators noted that nicotine produced a rise in cholesterol in male but not in female rabbits ³³ whereas in our study no sex differences were observed, albeit the numbers of each were few. Stefanovich et al. ²⁸ found slightly greater aortic atherosclerosis and serum cholesterol in nicotine, cholesterol-fed rabbits. It does appear significant that these investigators utilized

1003541871

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

J. M. Ramsey

100% - 2 summer
months
10% Academic
Year

REDACTED

Technical

Thomas M. Fitzsimmons

20% all year

REDACTED

Gregory MacNealy

Part-Time Lab Technicians

Staff Benefits

Sub-Total for A

REDACTED

B. Consumable supplies (by major categories)

2 Tanks, 100% Carbon Monoxide (Matheson), \$7.50 ea.

\$ 15

20 Vials Pre-Cal Hematocrit Tubes, \$2.50 ea.

50

200 Disposable Syringes and Needles

25

20 Vials, 20 Lambda Disposable Blood Pipets, \$13 ea.

260

Chemical Reagents

50

Sub-Total for B

\$ 400

C. Other expenses (itemize)

Remuneration for 10 Experimental Subjects, \$100 ea.

Sub-Total for C

\$ 1,000

Running Total of A + B + C

REDACTED

D. Permanent equipment (itemize)

S-51720 Centrifuge (Sargent)

\$ 120

2-675-150V1 Hemophotometer (Fisher)

300

Sub-Total for D

\$ 420

E

\$ 1,362

Total request

REDACTED

E. Indirect costs (15% of A+B+C)

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	\$422	\$1,200	0	\$1,458	REDACTED
Year 3	REDACTED	\$445	\$1,200	0	\$1,529	REDACTED

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1003541875

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1003541899

#864A - SLOTKIN

1003541933

8. Any additional facilities now required? Describe briefly:

We do not anticipate the need of additional major items of capital equipment.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Peter C. Moller, Ph.D., has joined our staff as a co-investigator. Dr. Moller replaces Dr. Doris Chang, who returned to Taiwan. Dr. Moller has experience in both electron microscopy and cell culture. He will assist with the electron microscopy required for the proposed program and will also have the primary responsibility for scaling up cultures of pulmonary endothelial cells.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

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(Continued on Page 2a)

12. Summary progress report (append in standard form as separate document, unless recently submitted).

1003541912

3.

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
*Una Ryan, Ph.D. Principal Investigator	25%	
**J.W. Ryan, M.D., D.Phil. Co-Investigator	10%	
**Peter C. Moller, Ph.D. Co-Investigator	50%	REDACTED
Fringe Benefits		

Technical

Sharon Monticone	Lab Tech II	100%	REDACTED
Erica Clements	Lab Tech	50%	
Fringe Benefits			

REDACTED

Sub-Total for A

B. Consumable supplies (by major categories)

Animals: rats \$400, rabbits \$200, Golden hamsters \$100	700
Photographic supplies	1,000
Chemicals and glassware, general	800
Fixatives, embedding and staining materials	700
Cell culture media, culture chambers and compress gas tank	1,200

Sub-Total for B 4,400

C. Other expenses (itemize)

Travel	
Una Ryan	500
J.W. Ryan	500
Peter C. Moller	500
Shipping charges	150
Reference Books	75
Regrinding diamond knives for microtomy	350

Sub-Total for C 2,075

Running Total of A + B + C

REDACTED

D. Permanent equipment (itemize)

Durst glassless negative carrier	80
Photographic easel	60

Sub-Total for D 140

E. Indirect costs (15% of A+B+C)

E 4,596

Total request

REDACTED

1003541914

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1003541876

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1003541879

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

May 30, 1973

Grant Application No. 910

To: The Committee comprising Drs. Bing, Gardner and Jacobson
Subject: James M. Ramsey, M.S.
New application No. 910
"The Effects of Chronic Exposure to Low Level Carbon Monoxide
on the Red Cell Mass and Blood Viscosity in Human Subjects"

History

An outline submitted earlier became Case No. 154; the Executive Committee after review encouraged full application.

Application No. 910 requests \$10,859 plus two additional years.

Document Submitted (attached)

Application dated 4 May 1973.

The four reprints marked with asterisks on page 9 of the application are available here, and will be sent to you on request.

Comment

The question of human subjects in a nonmedical setting is discussed on page 4. We have in file approvals of the responsible local "Advisory Committee for the Protection of Human Rights".

On page 8 of the application appears a comment concerning the principal investigator's lack of a "terminal" degree.

No outside opinion on this proposal will be sought unless you so request.

F.W.N.

FWN:wg
Encl.

1003541886

10. Experimental protocol for ensuing year.

The chronic smoking program will be carried out in litter mate beagles. After more than two years experience we have found this species quite tolerant of the chronic smoking program. The technique of chronic tracheostomy under anesthesia as described by Cahan and Kirman (3) is employed. After ten days of healing, the animals begin cigarette smoking by replacing the regular tracheostomy tube with a teflon tube coupled with latex tubing to the smoking machine which regulates the duration and volume of inhaled smoke. Two weeks are usually needed for the animal to adjust to the initial reaction and to smoking voluntarily. Both control and experimental animals will have tracheostomy and will be maintained in the same environmental conditions. Routine monitoring of weight, hematocrit and serum proteins is performed throughout the study. In addition, at intervals of three months, blood will be taken to assess clotting proteins, platelets and plasma lipids. Arterial blood pressure will be monitored in the awake but relaxed animal, to evaluate our previous observations of hypertension in the anesthetized beagle in a chronic smoking program. The animals will also have studies of ventricular conduction by high frequency precordial EKG (4) and ventricular function from the ejection times (5). These experiments will consider the following variables: the duration of smoking, the influence of high lipid diet and the effects of combined ethanol use.

Litter mate beagle dogs, one to two years of age, will be used. Each group will be divided into a smoking and nonsmoking group. It is intended to observe them at least two to three years. Group I: eight animals will be maintained on a standard canine diet without smoking and eight will smoke seven cigarettes per day with the same diet. Group II will consist of eight dogs placed on the standard diet plus 36% of calories as ethanol; the other half of the group will receive the same regimen combined with smoking.

a. Clotting and fibrinolytic studies: The following is a summary of the studies on coagulation and fibrinolytic activity carried out in our currently ongoing chronic smoking program.

- 1) Whole blood clotting time (Lee and White) in glass and plastic tubes in duplicate.
- 2) Partial thromboplastic time (Hicks and Pitman, Brit. J. Haematol. 3: 227, 1957).
- 3) Platelet counts--direct (Breckner, C., and Cronkite, E.J., J. Appl. Physiol. 3: 365, 1950).
- 4) Platelet adhesiveness (Saltzman, E.J., J. Clin. Med. 62: 74, 1963).
- 5) Plasma fibrinogen levels (Quick, A.J., Hemorrhagic Disease. Phila., Lee and Faberger, 1957, pp. 426-439).
- 6) Fibrinolytic activity by Euglobulin lysis time (Van Kaulla, K., and Schultz, R., Amer. J. Clin. Pathol. 29: 104, 1958) and on unheated bovine fibrin plates (method of Astrup and Muller, as modified by Holemans and Robers, J. Lab. Clin. Med. 64: 778, 1964).

1003541904

8. Brief statement of working hypothesis:

There is a statistical relationship between long-term smoking habit and cardiovascular disease, and carbon monoxide is one of the tobacco combustion products upon which speculation has centered as a potential factor in this relationship. The role of erythrocytic polycythemia as a potential adaptation to persistent hypoxia may not be an entirely advantageous adaptate. An increased erythrocytic concentration increases the blood viscosity, thereby increasing blood flow resistance. In addition to imposing additional work loads on the myocardium and possibly enhancing risks of thrombus formation, the increased flow resistance can be critical in view of the fact that an increased flow rate is the chief counter-effect in oxygenation of the myocardium when hypoxemia exists (in hypoxemic states other organs increase O₂ extraction, whereas the heart doesn't). In chronic hypoxic hypoxemia (hypobaric O₂) there is a tendency for the red cell mass to increase (polycythemia), and it is thought that a reduced arterial PO₂ triggers erythropoiesis. There is discrepancy in respect to human studies with carbon monoxide hypoxemia, and the relation between intermittent, chronic, low level CO exposures and hematological effects needs resolution. One major difference between the two types of hypoxemia is that with CO the arterial PO₂ is relatively normal, only the O₂ content is affected.

Many studies have indicated that animal exposure to decompression or altitude has resulted in reticulocytosis, erythrocytosis, and elevated hematocrit. Certain studies (Cavusoglu and Kayserilioglu) have shown that such exposures have not appreciably affected hemoglobin synthesis, which suggests that Hb synthesis and cell divisions may be controlled by different mechanisms. Often associated with the increased red cell mass is a plasma volume decrease (Reissmann). Furthermore, animals polycythemic from hypobaric hypoxia show a suppression of RBC production after returning to ambient pressures (Shadduck et al.).

Numerous studies of man at altitude or with decompression chambers likewise have shown a polycythemic response; the immediate effect believed due to a hemoconcentration and continued exposure resulting in elevation of red matter through erythropoiesis. Hurtado has shown that people living at altitude have greater blood viscosity and some macrocytosis of the RBC's. Merino, on the other hand, studying subjects at 4,000 meters, indicated a tendency of erythrocytic microcytosis and a lack of parallelism between increases in RBC and Hb, the former increasing more so. In addition, he found polycythemia to reverse itself when subjects were brought to sea level.

Sanchez studied human subjects at 4,000 meters and found significant elevations in hematocrit and reductions in plasma volume although the total blood volume increased. The polycythemia resembled that seen in patients with congenital heart disease. Billings et al., studied human subjects at 3,800 meters for 20 days. The mean hematocrit increased for two weeks, then stabilized for the remainder of the time.

In exposing animals to CO, some studies have shown elevation of Hb and hematocrit (Jones et al.). This was a continuous exposure (200 ppm for 90 days). Wilks et al., exposed seven dogs daily (six to eight hours) to 80 ppm CO and obtained increases in Hb content and hematocrit. Ramsey found increases in Hb and reticulocyte percentages in rats briefly exposed to high levels of CO (1200 ppm). However, other studies of intermittent and continuous exposures (50 ppm) with rodents and dogs have produced no hematological changes (Lindberg) (Stupfel et al.).

1003541888

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1003541906

In addition to these parameters, hypercoagulable state or thrombogenicity will be evaluated by kinetic studies of platelet and fibrinogen. Thus, the survival rates of ^{51}Cr tagged platelets (6) and ^{125}I tagged fibrinogen (7) will be determined from their disappearance rates in sequential samples taken after administration of the tracers (7). In addition, platelet function will be evaluated by estimating platelet aggregation. Platelet rich plasma aliquots will be exposed to standard doses of ADP, collagen or epinephrine, recording aggregation patterns by means of an aggregometer (8, 9). At the conclusion of the study, samples will be taken from the artery and coronary sinus for evaluating of clotting and fibrinolytic activity as well as evaluation of thrombogenicity by means of the Wessler technique. The myocardial and renal uptake of fibrinogen will be assessed. If indicated for localization, autoradiography will be performed.

b. Morphology: Since we have observed apparent accumulation of triglyceride in myocardial cells in chronic smoking beagles, myocardial specimens will be obtained by a catheter biopsy technique for light and electron microscopic examination at six months intervals. Following sacrifice of the dog or in case of sudden death, tissue specimens from heart muscle, coronary arteries, conduction system as well as aorta, will be subjected to extensive morphologic examination and the results will be correlated with those obtained from the hemodynamic and metabolic studies. Specimens of coronary artery, conduction bundle and myocardium are to be fixed in 10% neutral buffered formalin for routine microscopy. Sudan IV preparations are prepared on formalin fixed cryostat-sectioned material. Remaining tissues are processed and stained with hematoxylin eosin, Alcian Blue, PAS, Gormori's aldehyde-fuchsin, Van Gieson elastica and trichrome. Specimens for electron microscopy are cut into small sections fixed in cold-buffered glutaraldehyde, post-fixed in osmium, exposed to lead and uranyl acetate and then imbedded in epon. Sectioning is done on a Porter-Blum ultramicrotome and electron microscopy performed on a Siemens Elmskop I.

c. Ventricular function: The smoking periods will be concluded after 12, 24 or 36 months. Each dog will be anesthetized for hemodynamic studies with chest intact. The parameters include left ventricular systolic pressure and its first derivative, end-diastolic pressure and volume, cardiac output by the indicator dye dilution technique at rest and during afterload or preload increments, using angiotensin or rapid intraventricular infusion of normal saline; calculation of stroke volumes and stroke work as well as determination of ventricular contractility by the Frank-Levinson index (10). In addition, conduction in the His-Purkinje system and ventricular wall will be measured (11).

d. Myocardial metabolism: Coronary blood flow will be assessed by the ^{85}Kr clearance technique (12) before and during tachycardia induced with atrial pacing. Myocardial production of lactate (13) or leakage of potassium into the coronary sinus will be evaluated as an index of myocardial ischemia. Electronmicrographs will supplement the evaluation of ischemia. In addition, after a suitable recovery period, the heart will be cold-arrested and the transmural distribution of potassium, sodium, triglyceride and free fatty acid, since altered myocardial content has been observed during ischemia or catecholamine infusion (14).

1003541905

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested):

	% time	Amount
T.J. Regan, M.D.	15%	xxxxxx
C.B. Moschos, M.D.	20%	xxxxxx
M.M. Lyons, M.D.	15%	xxxxxx
S.S. Ahmed, M.D.	30%	xxxxxx
G. Manskopf, M.D.	20%	xxxxxx
H. Oldewurtel	10%	xxxxxx

Technical

F. Herdman	100%	6,736
R. DeSantis (diener)	75%	7,200
Pathology technician	45%	5,013
		<u>18,949</u>
Fringe benefits (17%)		3,221

Sub-Total for A 22,170

B. Consumable supplies (by major categories)

Animals: 30 litter mate beagles @ \$108 ea.	3,240
Animal maintenance, \$1/dog/day	10,950
Smoking apparatus, tracheostomies, cigarettes	275
Glassware, syringes	350
Reagents for coagulation & biochemical analyses	610
Special diet (high lipid)	780
Isotopes 125-I, 51-Cr, 85-Kr)	890
	<u>17,095</u>

Sub-Total for B

C. Other expenses (itemize)

Travel: domestic, attend scientific meetings	300
--	-----

Sub-Total for C 300Running Total of A + B + C 39,565

D. Permanent equipment (itemize)

Sub-Total for D

E. Indirect costs (15% of A+B+C)

E 5,935

Total request

45,500

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1003541927

LEGENDS

Fig. 1A. Coronary angiogram of untreated control revealing filling of right (R) and left coronaries. Filling of the circumflex (C) branch of the latter is greater than that of the descending (D) tributary.

1B. Angiogram from hypertensive, cholesterol-fed rabbit revealing foci of narrowing (arrows) in circumflex branch. The descending and right coronaries do not exhibit as much filling or tortuosity as noted in the control.

Fig. 2A. Selective left coronary angiogram in untreated control.

2B. The angiogram of hypertensive, cholesterol-fed rabbit discloses focal narrowing and irregularity (arrows) of circumflex and descending branches of the left coronary.

Fig. 3. Schematic presentation of atherosclerosis in aorta of (A) normotensive, cholesterol-fed rabbits with and without nicotine administration, (B) hypertensive, cholesterol-fed rabbits with and without nicotine, and (C) non-cholesterol-fed rabbits with hypertension and/or nicotine.

Fig. 4. Cross sections of extramural branches of coronary artery from (A) non-cholesterol-fed, nicotine-treated rabbit. The appearance is similar to that of untouched controls, (B) non-cholesterol-fed, hypertensive rabbit.

The luminal area is larger than that noted in A, and (C) hypertensive, cholesterol-fed rabbit revealing intimal cushion and plaque. Orcein elastica X82.

Fig. 5. Appearance of lipid cushions occluding lumens of intramural coronary branches of cholesterol-fed rabbit. H&E X 150.

Fig. 6. Miliary necrosis of myocardium in hypertensive, cholesterol-fed

12. Biographical Sketch

Name: JAMES M. RAMSEY
 Title: Principal Investigator
 Associate Professor of Biology,
 University of Dayton - Environmental
 Physiologist

Age:

REDACTED

Education:

B.S. degree, Wilmington College
(Zoology), 1948;

M.S. degree, Miami University
(Physiology), 1951;

Additional graduate study, University
of Cincinnati (Environmental Toxicology),
1957-1959.

Because of my research reputation,
everyone presumes I have terminal
degree; which I don't.

Experience:

Associate Professor, Department of Biology, University
of Dayton, 1970;

Assistant Professor, Department of Biology, University
of Dayton, 1964;

Medical Research Associate, University of Cincinnati
College of Medicine, 2 years;

Instructor and Research Associate, Physiology, Miami
University, Oxford, Ohio, 5 years;

Instructor, Biology, Cedarville College, Cedarville,
Ohio, 4 years

Professional Membership

Affiliations:

REDACTED**REDACTED**

Listed

Recognition:

American Men of Science; Dictionary of International
Biography

Past Research
Grants:

National Science Foundation, Research Related to
Carbon Monoxide Toxicology, July 1966-1967, \$1,600;

National Science Foundation, Research Related to
Carbon Monoxide Toxicology, July 1967-1968, \$2,000;

Public Health Service (NAPCA), Research Related to

Carbon Monoxide Toxicology, July 1968-Nov. 1969, \$11,037;

1003541894

CURRICULUM VITAE

Name: Peter Christian Moller

Birth date and place:

REDACTED

Marital Status:

REDACTED

Education and Degrees:

University of Houston, Houston, Texas -- 1965 -- B.Sc. Biology
Rice University, Houston, Texas -- 1971 -- Ph.D. Cell Biology

Thesis Advisor: Dr. Charles W. Philpott

Thesis Title: The Pharyngeal Circulatory System of Amphioxus:
Fine Structure and Cytochemical Studies of the
Vascular System in a Cephalochordate.

Research and/or Professional Experience:

1965-1967	Chief Technician, Cell Biology and EM Laboratory, Department of Biology, Rice University, Houston, Texas.
1967	Instructor in Introduction to Biology, Rice University, Houston, Texas.
1968	Instructor in Botany Laboratory, Rice University, Houston, Texas.
1969	Instructor in Cellular Physiology Laboratory, Rice University, Houston, Texas.
1971 (summer)	Postdoctoral Fellowship, Department of Biology, Rice University, Houston, Texas (With Dr. J.W. Campbell).
9/71-5/73	Research Fellow, Division of Biological and Medical Sciences, Brown University, Providence, Rhode Island (With Dr. Richard A. Ellis).
6/73-present	Research Scientist, Papanicolaou Cancer Research Institute, Miami, Florida.

Military Service:

1957-1959 United States Coast Guard

Academic and Professional Honors:

Fellow-Trainee, U.S.P.H.S., Rice University, 1967-1971.
Postdoctoral Fellowship, U.S.P.H.S., Rice University, 1971 (summer).
Postdoctoral Fellowship, U.S.P.H.S., Brown University, 1971-1973.

1003541931

determinants of ASHD as hypercholesterolemia and/or hypertension.

MATERIALS AND METHODS

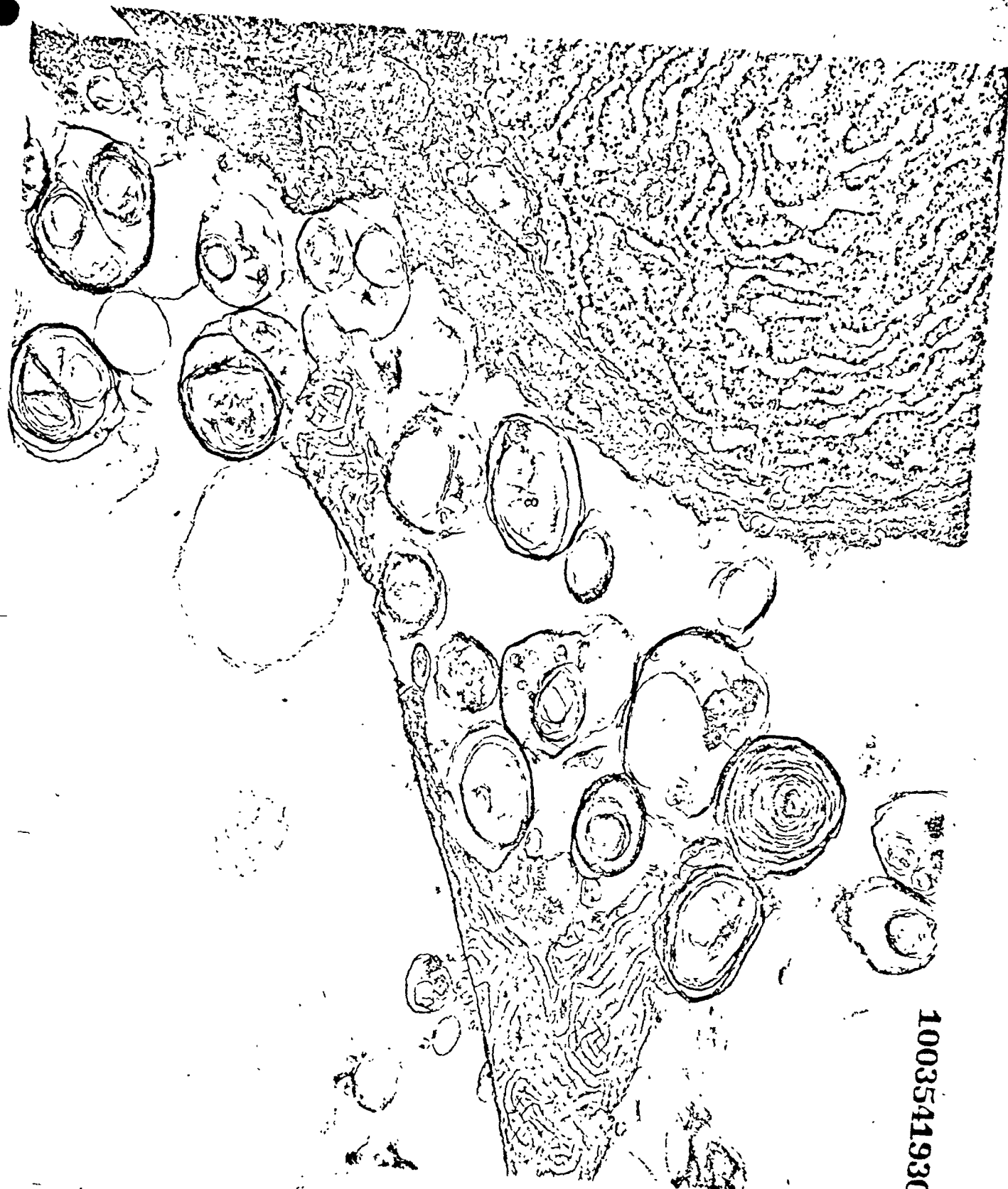
Eighty-seven adult male and female albino rabbits weighing 2-2.5 kg. survived or satisfied the requirements of the experiment. These comprised the following groups. Group I consisted of 16 that received Purina rabbit chow containing 2% cholesterol. Group II consisted of 10 that received the regular ration without added cholesterol but were given twice daily subcutaneous injections of 0.5 mg of nicotine dissolved in physiologic saline. Preliminary studies revealed that 0.5 mg. of nicotine caused a transient rise of 15-20 mm. Hg. in blood pressure and tachycardia in normal rabbits. This total daily dose is estimated on a weight basis to be equivalent to smoking approximately 35 cigarettes per day in man considering that 1 mg. of nicotine is absorbed from 1 inhaled cigarette.⁴⁷ There were 12 in Group III in which hypertension was successfully induced by the method of Page⁴⁸ except that both unilateral nephrectomy and celophane enclosure of the contralateral kidney were performed in one stage. Group IV consisted of 12 hypertensive rabbits that received nicotine as described above. Group V was comprised of 10 cholesterol-fed hypertensive animals and Group VI, 15 similarly fed normotensive rabbits that received nicotine as above. Group VII consisted of 12 hypertensive cholesterol-fed animals that also received nicotine.

Animals were sacrificed 90 days following operation and/or the administration of the cholesterol diet or nicotine injections.

1003541864

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- Weibel, E.R. and Gil, J.: Resp. Physiol., 4, 42, 1968.
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1003541928



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Counter. Reticulocyte percentages are obtained from thin smears with new methylene blue staining (Color Index 927-Wintrobe). Percentages from 15 different slide locations are averaged. Blood viscosity is determined by means of the viscosimeter of Hess, based on Poiseuille's law. The test requires only a drop of blood and can be performed in 30 seconds.

After three months of exposure to normal air (controls) and daily determinations of the hematological series in the eight subjects, all subjects are then subjected twice a day to the chamber in the very same manner except that now the chamber has a concentration of 300 ppm carbon monoxide in air. Everything else is identical to the first three months subjection to the chamber. The subjects do not know what they're breathing and the technicians do not know anything about the exposures (hematological determinations are performed at a different time and in a different room). Therefore, a double psychological blind exists throughout the study. 300 ppm CO for two periods of 45 minutes each should provide an accumulation of 5 to 8% carboxyhemoglobin each day (reversed each night). This is realistic with that found in heavy smokers by the end of a day. The chamber is loaded from a tank of 100% CO with two-stage regulator and precise flow meter setting to adjust the chamber interior to a desired concentration of CO. The chamber is exhaustable and can be cleared. I have had considerable experience with such exposure techniques with human subjects. Air in the chamber can be monitored (unknown to occupants) for CO concentration. The twice daily CO exposures are done each weekday for six months. Blood samples are taken for CO content and plasma volume determinations (same two subjects as during the first three months) as was described for the control runs. Also, the five days a week hematological series (Hb, hematocrit, RBC count, reticulocyte percentage, and blood viscosity) is determined each morning. After the six months of CO exposures, the hematological series and the plasma volume determinations are continued for an additional month.

The Biology Department has computer facilities so that data reduction and statistical analyses can be performed readily. Most of the tests applicable to the type data of the proposed study are parametric. Even though close attention will be given individually to the ten subjects, the results of the study must be viewed with considerable statistical inference. Eight to ten subjects should be sufficient in view of the fact that they are serving as their own controls, which reduces variance considerably. Therefore, much attention will be given to comparing means for each treatment and the testing for significance of mean differences with paired "t" values in before-after treatment on the same subject. Also, much of the data would lend itself to correlation-regression application; e. g., degree of hematological change with time, or correlating degree of change in one hematological parameter with that of another. Nonparametric (chi-square) procedures may be employed to evaluate whether or not subjects show change, irrespective of degree.

With no unforeseen difficulties, the data may certainly be worth publishing, perhaps in the Journal of Applied Physiology or "Blood".

Depending on the data obtained this first year, additional study may be in order to further elucidate points of the first year's data.

1003541891

Our first studies will use essentially the same protocol as that of Redding et al. (1972). Rats will be made hyperthyroid by daily subcutaneous injections of 1 mg L-thyroxine per kilogram for 6 days. Thyroid status will be monitored by assay of serum-protein-bound iodine and by following body weight. We will incorporate additional studies using triiodothyronine. In addition, studies on the absence of hormone will be accomplished using rats 6-8 weeks after thyroidectomy. We will coordinate the morphologic studies with studies of lung lavage fluid as described by Redding et al., 1972. Studies of fine structure of the giant alveolar cells and their lamellar inclusions will be conducted as described above (freeze-etch replicas and thin sections), while monitoring Clara cells and macrophages.

Synthetic glucocorticoids may well accelerate maturation of giant alveolar cells (Olsen, 1972). In addition, it is known that the lungs take up as much aldosterone as do the kidneys (Sulya et al., 1963). Therefore we propose to conduct a similar series of investigations incorporating controls, adrenalectomized animals and adrenalectomized animals with specific replacement therapy (cortisol, corticosterone, desoxycorticosterone or aldosterone).

The effects of inhaled nicotine may well be of interest in terms of the fine structure of giant alveolar cells and in terms of the fate of lamellar bodies. As described previously, Olsen (1972) has shown that pilocarpine causes acute depletion of lamellar bodies of giant alveolar cells while increasing the phospholipid content of lung lavage fluid. These effects are prevented or partially inhibited by atropine, a point suggesting that the pilocarpine effect is muscarinic (in terms of the classification of acetylcholine-like effects). Possible nicotinic effects have not been tested, but could be of importance in terms of direct cellular effects or parasympathetic synaptic effects.

Chlorphentermine may present another opportunity. If, as existing data indicate, chlorphentermine stimulates the rate of synthesis of lamellar bodies, chronic administration of the drug to experimental animals should enhance our ability to identify the major stages of genesis, maturation and secretion of these inclusions.

Although it is highly-speculative at the moment, it is possible that chlorphentermine interferes with cholesterol synthesis. Dr. Lüllmann-Rauch (personal communication) has found that triparanol, a well-studied inhibitor of cholesterol synthesis, also induces the accumulation of airspace "foam" cells and of free lamellar forms. This point can be considered with the findings by Werb and Cohn (1972) which indicate that membrane synthesis requires exogenous cholesterol. Possibly, when cholesterol is not available, other membrane components, such as phospholipid, accumulate in excess.

1003541922

Theodore A. Slotkin - Privileged Communication

Publications from this Project

1. Maturation of the adrenal medulla. I. Uptake and storage of amines in isolated storage vesicles of the rat. T.A. Slotkin, Biochem. Pharmacol. in press.
2. Maturation of the adrenal medulla. II. Content and properties of catecholamine storage vesicles of the rat. T.A. Slotkin, Biochem. Pharmacol. in press.
3. Binding of amines to purified bovine adrenal medullary storage vesicle membranes. T.A. Slotkin and N. Kirshner. Biochem. Pharmacol. in press.
4. Secretion and recovery of catecholamines from the adrenal medulla. N. Kirshner and T.A. Slotkin, Biochem. Pharmacol. in press.
5. Hypothetical model of catecholamine uptake into adrenal medullary vesicles. T.A. Slotkin, Life Sci. in press.
6. Reserpine-like effects of harmine on adrenal medullary storage vesicles. H.O. Green and T.A. Slotkin, submitted for publication.

Abstracts:

1. T. A. Slotkin, Uptake and storage of amines in isolated adrenal medullary vesicles of developing rats. Fed. Proc. 32: 783 Abs. (1973).
2. T. A. Slotkin, Maturation of Adrenal Catecholamine Storage Vesicles of the Rat. Pharmacologist, in press.

1003541948

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

August 6, 1973

Grant Application No. 814R2
CARDIOVASCULAR

To: The committee comprising Drs. Bing, Loosli, Sommers,
and Wyatt

Subject: Una S. Ryan, Ph.D., Papanicolaou Cancer Research
Institute, Miami
Second renewal No. 814R2
"The Role of Endothelial and Epithelial Cells in Non-
Ventilatory Functions of the Lungs"

History

This grant began in 1971; subsequently Dr. Smith transferred from the Department of Medicine at the University of Miami School of Medicine to her present location.

The current grant is for \$32,908. Application No. 814R2 requests \$35,373 for the third and final year of an approved three year plan. The initial estimate for this year was \$26,235.

Documents Submitted

Attached is application dated July 30, 1973 with C.V. of Dr. Peter C. Moller.

Comment

With this renewal, the principal investigator and co-investigator have become a husband and wife team.

F.W.N.
F.W.N.

FWN:wg
Encls.

1003541910

Ten young, male human subjects of the same age would be recruited from a selective screening process. The subjects must be healthy and nonsmokers. Included in the screening process would be examinations for pulmonary performance, EKG, and standard hematology. Permission from each selectee's personal physician is also obtained. The subjects are paid for their services. I have used human subjects in several past studies and am aware of necessary precautions. The University of Dayton has a board for evaluating all research proposals involving human subjects. The subjects in the proposed study will likely be University students. An effort will be made to select subjects whose hematology (RBC count, Hb, hematocrit) shows a stable day by day level, and it is also quite desirable that there is uniformity (slight standard deviation) within the group in respect to hematological values. Rather than use two groups of subjects for exposed subjects and controls, respectively, I shall employ just one group and let them be their own controls. In this way there is a perfect match or comparison of exposed versus unexposed effects. By using the ten subjects first as controls, this design is quite feasible and therefore most desirable. Throughout the duration of the study the subjects must adhere to a reasonable dietary and drinking regime, and must possess daily and week-end habits that prevent any prolonged or frequent sojourns in automobile traffic.

The first seven weeks of the study would be spent in recruiting, screening, and selecting subjects as well as obtaining adequate supplies and performing preliminary operations.

Beginning with the eighth week, data recording would begin. The ten subjects would be subjected twice daily (45 minutes each time) to normal air in our large, walk-in, environmental chamber. During the time in the chamber, the subjects may read, play cards, or otherwise leisurely pass the time. They are never told at any time about the composition of gaseous material they breathe. Exposure to normal air twice daily each weekday would continue for three months. Every third day, 2 ml of antecubital vein blood is withdrawn from each of two subjects immediately upon leaving the second session in the chamber. Each time it is a different two subjects until it cycles around. This blood is examined for carbon monoxide by the method of Trinder and Harper, excellent for low ranges. Also, every fourth day, plasma volume determinations are performed on each of two subjects. This is done with antecubital vein blood employing the Evans dye method. This is performed on the same two subjects each time and since 10 ml of blood is required, these two subjects are not used for other hematological evaluations. Also, because of this volume of blood and potential dye retention for two to three days, the determination is done every fourth day. It is performed in the morning (fasting). Also, for five days each week, a battery of hematological evaluations are performed on each of the other eight subjects. These are done at the same time each day (morning with fasting conditions) and are determined from microquantities of capillary blood from finger prick. Less than 0.4 ml of blood is required for the entire series and since microquantities are involved, most of the determinations are done in triplicate. Determinations for Hb content are done with the cyanmethemoglobin method and read spectrophotometrically. Hematocrits are determined with the International Micro-Capillary Centrifuge, Model MB, and the accessory micro-capillary reader, Model CR. Erythrocyte counts are determined with a Coulter

p.9

Theodore A. Slotkin - Privileged Communication

CURRICULUM VITAE

NAME:

Theodore Alan Slotkin

BORN:**REDACTED**MARRIED:**REDACTED**CHILDREN:**REDACTED**SOCIAL SECURITY NUMBER:**REDACTED**EDUCATION AND DEGREES:

B.S. - Brooklyn College, CUNY, 1967

Ph.D. - Department of Pharmacology and Toxicology - Univ. of Rochester, 1970

POSITIONS HELD:

June 1971 - present

Assistant Professor, Dept. of Physiology and
Pharmacology, Duke Univ. Medical Center

June 1970 - May 1971

Research Fellow, Dept. of Biochemistry,
Duke Univ. Medical Center

February 1970 - May 1970

Postdoctoral Fellow, Dept. of Pharmacology
and Toxicology, Univ. of RochesterSOCIETIES:**REDACTED**RESEARCH ACTIVITIES:

Neurochemistry

Neuropharmacology

1003541943

#833A-WELTMAN

1003541951

reticulum may have the effect of "floating" the surface lipid. On this point, ruthenium red has been used in previous studies of airspace contents (Brooks, 1969), but without the benefit of an embedding medium which preserves lipid membrane. Ruthenium red both stains and precipitates acidic polysaccharides, and therefore there should be no major losses during aqueous embedding.

Freeze-etch studies. The major effort in further freeze-etch studies is to extend our previous findings of lamellar organization and substructure of the leaflets in a manner to allow precise measurements. It should be emphasized that while freeze-etching can produce an elegant demonstration of structure and substructure, one cannot control exactly where fractures will occur. The only feasible approach to obtaining a full range of fracture planes is to make large numbers of replicas using large numbers of different tissue blocks prepared by several different means (high glycerol content, low glycerol, etc.). Clearly, this approach requires considerable time. As we described under Technical developments (above), the further development of aqueous embedding media will also take a great deal of time. We therefore plan to start making replicas immediately so that, as suitable thin sections become available, coordinated studies may proceed apace. This need not involve a hazard in deterioration of material as the metallic freeze-etch replicas are relatively durable.

We plan to find means of examining the unfractured membrane surfaces of giant alveolar cells and of lamellar bodies. For these experiments, glycerol, the usual cryoprotectant, will be omitted or reduced to low concentrations to permit lowering of the water table by sublimation. Etching will be continued for up to 10 minutes. Visualization of membrane surfaces as well as their fracture faces may allow a further understanding of the disposition of the globular particles presumed to be intramembranous (Branton, 1971), and their possible relationships to the release of lamellar inclusions. In other systems organization of intramembranous particles appears to be intimately related to membrane fusion and exocytosis (Satir et al., 1971; Smith et al., 1973).

As mentioned above, replicas are of uncertain thickness and therefore one cannot be certain of the validity of measurements derived from what is, in fact, a coating of the original structure. However, it now appears feasible using a film thickness monitor which has just become available (Balzers, Lichtenstein) to make replicas of reproducible thickness, thus making it possible to compare one replica with the next even though absolute measurements may be in doubt. Comparative measurements can then be made between the periodicity of extracellular tubular myelin and the substructural array of particles on lamellar body leaflets. Measurements of the spacing of tubular myelin in thin sections show a 400Å-500Å lattice which corresponds with the freeze-etch repeating pattern. Furthermore, it is important to establish the interlamellar distance, which on the basis of our present measurements, appears to be characteristic for this organelle and may be a function of chemical composition.

Relationship of lamellar bodies to other organelles of the giant alveolar cell. The close relationship between lamellar bodies and mitochondria has been illustrated by our pilot study. However, other laboratories have suggested that lamellar bodies arise in association with multivesicular bodies (Vatter et al., 1968), in the "cytoplasmic vesicle" (Hatasa and Nakamura, 1965) and in mitochondria

1003541920

entire preparation (cellulose acetate support and endothelial monolayer) as a vital chromatographic system such that angiotensin I was applied to the top of the support and angiotensin II was collected in the eluate. These data were presented at a colloquium "Protides of the Biological Fluids" in Brugge, Belgium (Ryan and Smith, 1973).

These results, in combination with our earlier findings that the plasma membrane/caveolae fraction of whole lung homogenates metabolized bradykinin, angiotensin I and the adenine nucleotides as does whole lung (Ryan and Smith, 1971), and that 5'-nucleotidase activity is localized in endothelial caveolae, all point to the endothelial plasma membrane as the site of enzymic activity. We have summarized this work in a review to the American Physiological Society, September, 1972, to be published: Smith and Ryan, Fed. Proc., 1973.

As discussed in the proposal of research submitted last year, we undertook studies on the fine structure of pulmonary endothelial cells. We found, as has been described for other cells studied by freeze-fracture techniques, that intramembranous particles (70-100Å in diameter) are distributed randomly on both fracture faces of undifferentiated plasma membrane. However, the particles organize in rings and plaques at the base of caveolae and appear to adhere predominantly to the outer leaflet. The specific organization of particles in association with caveolae confirms the presence of a skeletal supporting rim to caveolae, a point previously suggested by our studies of thin sections (Smith and Ryan, 1972). We also identified particles in association with the outer leaflet of the caveola membrane itself which may represent the globular substructures which we reported in thin-sectioned material and which we regard as likely candidates for enzyme clusters. Our freeze-fracture studies raise the question as to whether there is a relationship between the organization of intramembranous particles and the structure of endothelial caveolae. Whether the particles can organize as rings or plaques in response to stimuli and thus be related to the mechanism of initiation of pinocytosis is an intriguing possibility.

The organization and preferential adherence of intramembranous particles of pulmonary endothelial cells may well be germane to our studies of the localization of enzymes which metabolize circulating vasoactive substances. As will be discussed more fully in section 10 (Proposed research), Dr. F. Dorer and colleagues at the Cleveland Veterans Administration Hospital have isolated converting enzyme from hog lungs. The molecular weight of this enzyme is approximately 300,000. Assuming a globular configuration, an enzyme of this size could readily be accommodated in a particle 70-100Å in diameter. Dr. Dorer has made the enzyme available to us and descriptions of its use in our proposed experiments appear in greater detail in section 10.

Type II alveolar cells and surfactant

Part of the commitment of our current research is to study the normal structure and function of type II alveolar cells and their relationships to the surface active lining of the lung. The long-term aim of these studies is to provide a data base for understanding the effects of hormones and drugs (such as nicotine) on type II cells and for understanding the role of these cells in the processing of surfactant and possibly inhalants.

1003541924

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☐

Second Renewal ☒

Date: July 30, 1973

1. Principal Investigator (give title and degrees).

Una S. Ryan, Ph.D. (formerly Una Smith), Senior Scientist,
Papanicolaou Cancer Research Institute and
Assistant Professor of Medicine, University of Miami School of Medicine

2. Institution & address:

Papanicolaou Cancer Research Institute
1155 N.W. 14th Street
Miami, Florida 33136

Mailing Address: P.O. Box 6188
Miami, Florida 33123

3. Department(s) where research will be done or collaboration provided:

Cardiopulmonary Unit, Sieron Building
1425 N.W. 10th Avenue
Miami, Florida 33136

4. Short title of study:

THE ROLE OF ENDOTHELIAL AND EPITHELIAL CELLS IN
NON-VENTILATORY FUNCTIONS OF THE LUNGS

5. Proposed renewal date: January 1, 1974

6. How results to date have changed earlier specific research aims:

In general, our studies have produced few unexpected results in terms of the pulmonary processing of angiotensin I, bradykinin and the adenine nucleotides. In consequence, our specific aims have changed to allow a progressively finer focus on the precise subcellular localizations of the relevant enzymes. As indicated in our previous application, we have broadened the scope of the research program to include studies of type II alveolar epithelial cells, their lamellar inclusions and the modulations produced by hormones and drugs. We propose to continue these studies. However, in the previous application, we outlined experiments to examine the subcellular sites of pulmonary enzymes capable of degrading prostaglandins. To date, it has not been possible to perform the indicated experiments as we deliberately chose to pursue what we regarded as more promising leads in our studies of endothelial cells.

7. How results to date have changed earlier working hypothesis:

As in our previous applications, we intend to continue testing the hypothesis that pulmonary endothelial cells are actively engaged in the metabolism of circulating angiotensin I and bradykinin. The major focus for the coming year will be to determine the precise subcellular localization of angiotensin I converting enzyme by cyto-immunologic techniques using monospecific antibodies to hog lung converting enzyme.

The second major research goal is to examine the fine structure of type II alveolar cells and to examine the modulations of these cells and their lamellar inclusions by hormones and drugs such as nicotine.

1003541911

Dr. Bing
Dr. Gardner
Dr. Jacobson

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Re: Your Case #154

Application for Research Grant
(Use extra pages as needed)

MAY 11 1973

Date: 4 May 1973

1. Principal Investigator (give title and degrees):

James M. Ramsey, Associate Professor of Biology

B.S. degree, Wilmington College (Zoology)

M.S. degree, Miami University (Physiology)

Additional graduate study, University of Cincinnati (Environmental Toxicology)

2. Institution & address:

University of Dayton

300 College Park Avenue

Dayton, Ohio 45469

3. Department(s) where research will be done or collaboration provided:

Biology Department

University of Dayton

4. Short title of study:

The Effects of Chronic Exposure to Low Level Carbon Monoxide on the Red
Cell Mass and Blood Viscosity in Human Subjects

5. Proposed starting date: 1 July 1973

6. Estimated time to complete: One Year

7. Brief description of specific research aims:

Tobacco smoking habits subject individuals daily to intermittent, low level carbon monoxide exposure. Can the slight hypoxemia characteristic of small percentages of persistent carboxyhemoglobin tend to induce acclimation by altering the hematological capacity for oxygen transport?

The major objective of this proposed study is to evaluate whether or not long-term, daily experimental exposures to concentrations of carbon monoxide realistic with those confronting smokers will result in significant polycythemia and increases in blood viscosity in selected human subjects.

Lesser objectives include (a) some elucidation of potential mechanisms of hematological response, e. g., changes in plasma volume and erythropoietic activity; (b) whether or not rates of red cell production and hemoglobin synthesis appear to be closely correlated with each other in response to the stress; and (c) determining the course of hematological changes with time, including whether there is reversibility of incurred changes after exposures have ceased.

1003541887

We have undertaken a study in which we coordinated examination of thin sections with that of replicas of freeze-fractured material (D.S. Smith et al., 1972; U. Smith et al., 1973). Studies of thin sections confirmed previous reports that lamellar bodies are released into the alveolar space where they appear to disassemble to yield tubular myelin, a component of surfactant (see micrograph). Freeze-etch replicas reveal the intracellular lamellar bodies as highly structured, in which lamellae are arranged concentrically or in parallel rows. Fractured lamellae bear particles approximately 100Å in diameter which are remarkable for their organization in an array of parallel rows and ribs exactly resembling the periodicity (400-450Å) of the lattice of tubular myelin as it occurs in the airspace. We have suggested that the ribs exposed within the lamellar body may represent tubular myelin elements in the course of assembly (D.S. Smith et al., 1972; Smith and Ryan, 1973). Myelin and synthetic lamellar phase phospholipids invariably fracture to yield smooth surfaces (Deamer et al., 1970; Branton, 1971). The fracture faces of the lamellar body resemble other freeze-fractured biological membranes in containing intramembranous particles. Following the correlation made by Branton (1971), the presence of abundant particles may reflect corresponding physiological activity. Some fracture planes reveal incomplete rows of particles and suggest an entirely new set of morphological criteria for evaluating the maturation of lamellar bodies in addition to supporting the view that a component of surfactant is synthesized within the characteristic inclusions of the type II alveolar cell, prior to its exocrine release into the airspace.

Related Studies

Although the metabolism of bradykinin in the pulmonary circulation results in a complete inactivation, the metabolism of angiotensin I is in fact primarily an activation reaction. The major polypeptide metabolite of angiotensin I is angiotensin II, the most potent hypertensive agent known. In addition to its effects on the tone of peripheral vascular beds, angiotensin II is considered to be a highly selective secretagogue capable of stimulating the release of aldosterone from the adrenal cortex and catecholamines from the adrenal medulla (Laragh et al., 1960; Feldberg and Lewis, 1964). Thus it appears likely that the lungs, by processing angiotensin I, can influence specific activities of the adrenal gland.

Over the past year, we carried out a collaborative study with Dr. Hans Winkler, University of Innsbruck, on the effects of angiotensin II on the adrenal medulla and on the mechanism of release of catecholamines from medullary chromaffin cells. The effects of angiotensin II on the medulla are dramatic. Feldberg and Lewis (1964) have estimated that one molecule of angiotensin II may cause the release of 5,000 molecules of epinephrine. Over the past ten years, data has accrued indicating that epinephrine is contained in membrane-limited granules and is released from chromaffin cells by a process called exocytosis. The concept of exocytosis was based largely on morphological descriptions and the biochemical evidence that the contents of chromaffin granules are released to the extracellular space in the absence of a concomitant release of cytoplasmic substances or of membrane components (Smith and Winkler, 1972).

Using freeze-fracture techniques, we have demonstrated pockets indicating points of attachment and fusion of chromaffin granules with the plasma membrane.

1003541925

Theodore A. Slotkin - Privileged Communication

10. Space and facilities available (when elsewhere than item 2 indicates, state location): Laboratory consists of 850 sq. ft. fitted with standard laboratory-type benches. Major items of equipment include Sorval RC-ZB centrifuge, Beckman L5-50 ultracentrifuge with rotors, Farrand ratio fluorometer, catecholamine autoanalyzer, Wang 600-6-T-P programmable calculator, incubation bath, pH meter, balances and general items of glassware and hardware. Research facilities and liquid scintillation spectrometer.

11. Additional facilities required: None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s), append list, and provide reprints if available).

1003541942

Membership in Scientific Societies:

REDACTED

REDACTED

Presentations:

- October 1968 - Presentation to the Texas Society for Electron Microscopy.
- January 1969 - Demonstration to the Texas Society for Electron Microscopy (by invitation).
- April 1970 - Presentation to the American Association of Anatomists, Chicago, Illinois.
- May 1971 - Presentation to the Division of Biological and Medical Sciences, Brown University (by invitation).
- May 1971 - Presentation to Dr. S.S. Spicer's group, Department of Pathology, Medical University of South Carolina.
- December 1971 - Presentation to the Department of Zoology, University of Rhode Island (by invitation).

Abstracts:

- November 1971 - The circulatory system of Amphioxus, Abstract, The Eleventh Annual Meeting of the American Society for Cell Biology, New Orleans, Louisiana.

Publications:

- 1) Moller, P.C. and Philpott, C.W.: The circulatory system of Amphioxus (Branchiostoma floridae) I. Morphology of the major vessels of the pharyngeal area. J. Morph., 139:389-406, 1973.
- 2) Moller, P.C. and Philpott, C.W.: The circulatory system of Amphioxus II. Uptake of exogenous proteins by endothelial cells. Z. Zellforsch., in press.
- 3) Moller, P.C. and Ellis, R.A.: The excretory system of Amphioxus. Submitted to Am. J. Anat.

1003541932

Item #8. Brief Statement of Working Hypothesis

doses of nicotine than the Sprague-Dawley strain. Nicotine lowered the blood pressure readings of Sprague-Dawley renal hypertensive rats (32).

The capacity of nicotine to diversely effect blood pressure via the sympathetic and/or parasympathetic system, certain vascular chemoreceptors, and/or ganglionic blockade renders it difficult to completely differentiate between neurogenic and hormonal mediating influences on blood pressure regulating mechanisms.

Correlative studies of alterations in plasma glucose, Na^+ and K^+ as well as a detailed assay of the blood lipid profile (cholesterol, FFA, triglycerides and phospholipids) at various time and age periods should contribute knowledge concerning the fundamental basis and development of essential hypertension, cardiovascular diseases and associated pathologies, etc.

1003541963

#923 - BODEN

1003541984

16. Other sources of financial support.

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The Effect of Chronic, Low Level Carbon Monoxide Exposure on the Erythron of the Rat. (Graduate student thesis as part of study.)	University of Dayton	\$2,825	November 1972-1973

PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
	NONE		

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made.

Checks payable to

C. J. Ramsey, University of Dayton

Mailing address for checks

300 College Park Avenue

Dayton, Ohio 45469

Principal investigator

Typed Name James M. Ramsey

Signature J. M. Ramsey Date Apr. 9, 1973

Telephone (513) 229-3011
Area Code Number Extension

Responsible officer of institution

Typed Name Joseph W. Stander

Title Authorized Representative

Signature Joseph W. Stander Date 4/1/73

Telephone (513) 229-2246
Area Code Number Extension

1003541897

Item # 7. Brief Description of Specific Research Aims

As indicated in the progress report, both the SH and NH groups revealed accompanied by significant alterations in carbohydrate metabolism (72,73,75) (i.e., lower plasma glucose and liver glycogen levels). Although total plasma protein levels were significantly reduced due to depressions in $\alpha 1, \alpha 2$, beta and gamma globulins, albumin levels were significantly higher (72).

In the budget for permanent equipment etc., we have listed items such as thin layer chromatography and furnace-ashing oven apparatus. If these items are granted, the TLC equipment plus our existing tools will enable us to determine plasma and adrenal disoxycorticosterone levels for assay of mineralocorticoid output in the nicotine treated and control spontaneously hypertensive and normotensive animals.

In addition, the TLC equipment will enable us to extract and assay nicotine and metabolites of nicotine, i.e. cotinine from the treated animals.

The ashing oven will enable us to do PBI studies and measure thyroid function and activity in the test and control SH and normotensive groups.

1003541962

Theodore A. Slotkin - Privileged Communication

parameters which previously demonstrated differences, and as compared since

15. L.J. Ignarro and F.E. Shedeman, *J. Pharmacol. Exp. Ther.* **159**: 38 (1968).

16. F.W. Heggeness, J. Diliberto and V. DiStefano, *Proc. Soc. Exp. Biol. Med.* **133**: 1413 (1970).

17. L.L. Iversen, J. de Champlain, J. Glowinski and J. Axelrod, *J. Pharmacol. Exp. Ther.* **157**: 509 (1967).

18. B. Hokfelt, *Acta Physiol. Scand.* **25**: Suppl. 92 (1951).

19. R.L. Patrick and N. Kirshner, manuscript submitted.

20. S. Daikoku, O. Takashi, A. Takahashi and M. Sako, *Tokushima J. Exp. Med.* **16**: 153 (1969).

21. L.G. Elfvig, *Ultrastructures* **17**: 45 (1967).

22. W.J. Louis, R. Tabei, S. Spector and A. Sjoerdsma, *Circ. Res.* **24**: Suppl. 1, 93 (1969).

23. J. de Champlain, L. Krakoff and J. Axelrod, *Circ. Res.* **24**: Suppl. 1, 75 (1969).

24. T.C. Westfall, *Eur. J. Pharmacol.* **10**: 19 (1970).

25. A.W. Stott and R. Robinson, *Clin. Chim. Acta* **16**: 249 (1967).

26. M. Mendlowitz, R.L. Wolf and S.E. Gitlow, *Am. Heart J.* **79**: 401 (1970).

27. A.D. Smith and H. Winkler, *Biochem. J.* **103**: 480 (1967).

28. O.H. Viveros, L. Arqueros, R.J. Connett and N. Kirshner, *Mol. Pharmacol.* **5**: 69 (1969).

29. B.L. Strehler and J.K. Totter, in "Methods of Biochemical Analysis" vol. 1 (D. Glick, ed.) p. 344, Interscience Publishers, N.Y. (1954).

30. O.H. Viveros, L. Arqueros and N. Kirshner, *Mol. Pharmacol.* **5**: 342 (1969).

31. O.H. Viveros, L. Arqueros, R.J. Connett and N. Kirshner, *Mol. Pharmacol.* **5**: 60 (1969).

32. P. Lundborg, *Acta Physiol. Scand.* **67**: 432 (1966).

33. P. Lundborg and R. Sitzel, *Brit. J. Pharmacol. Chemother.* **29**: 342 (1967).

34. A.D. Smith, in "The Interaction of Drugs and Subcellular Components in Animal Cells" (P.N. Campbell, ed.) p. 239, Churchill, London (1968).

35. O.H. Viveros, L. Arqueros and N. Kirshner, *Mol. Pharmacol.* **7**: 444 (1971).

1003541941

8. Brief statement of working hypothesis (continued):

for secretin (10). This assay enables us to directly study the effects of nicotine or cigarette smoke on circulating levels of immunoreactive secretin under basal and HCl stimulated conditions.

1003541988

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

July 31, 1973

Grant application #864A
CARDIOVASCULAR

To: The committee comprising Drs. Bing, Gardner and Loosli

Subject: Theodore Alan Slotkin, Ph.D., Duke University, N.C.
Continuation Application #864A (no commitment)
"Maturation of the Adrenal Medulla: Catecholamine stores
in normal and hypertensive rats"

History

Grant #864, for the year 1973, was awarded in the amount requested (\$12,110.) without assurance of continued support.

Application #864A (which competes as a "continuation" without commitment) requests \$13,346. The original estimate for this year was \$12,787.

Documents Submitted (attached)

1. Application dated July 24, 1973 (16 pages).
2. Progress Report #1, January 1, 1973 - June 30, 1973.

Comment

Copies of the publications listed on p. 3 of the Progress Report will be sent to you if you wish.

FWN:gh

Attachment

F.W.N.
F.W.N.

1003541934

Theodore A. Slotkin - Privileged Communication¹⁵

14. First year budget:

A. Salaries (give names or state "to be recruited")

% time

Amount

Professional (give % time of investigator(s)
even if no salary requested)

Theodore A. Slotkin

60

Hannah Green

100

Fringe Benefits @ 12.10%

REDACTED

Technical

Sub-Total for A

REDACTED

B. Consumable supplies (by major categories)

Rats - 1000 @ \$2.00

2,000

Animal housing and shipping

500

Isotopes

1,500

Chemicals and hardware

1,000

Sub-Total for B

\$ 5,000.00

C. Other expenses (itemize)

Equipment maintenance and service

500

Travel to FASEB and ASPET meetings

500

Sub-Total for C

\$ 1,000.00

Running Total of A + B + C \$ REDACTED

D. Permanent equipment (itemize)

Sub-Total for D

E. Indirect costs (15% of A+B+C)

E

\$ 1,741.

Total request

REDACTED

1003541949

15. Estimated future requirements.

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2						
Year 3						

#905 - DOMINO

1003541998

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Endothelium: Structure and Functions (salary only)	American Heart Association (72 160)	15,000 (02) 16,000 (03) 17,000 (04) 18,000 (05)	July 1, 1973 to June 30, 1976
*Studies on Normal Lung Cell Separation, Culture and Morphology	National Heart and Lung Institute N01-HL-3-3015-LD	164,900 (of which \$97,000 is designated for the purchase of a Philips 301 electron microscope)	July 1, 1973 to Sept. 30, 1974

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
It is not yet known whether renewal funds will be available for the NHLI contract program (N01-HL-3- 3015-LD). If such funds become available, renewal will be requested.			

*The work solicited by the contract of the National Heart and Lung Institute does not overlap in approach, concept or timing with the research program described in the present application. However, the EM 301 electron microscope will be available to the studies proposed here for funding by the Council of Tobacco Research.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Una S. RyanSignature Una S. Ryan Date 7/30/73Telephone 305 373-5903
Area Code Number Extension

Responsible officer of institution

Typed Name Dr. Julius SchultzTitle Director and PresidentSignature Julius Schultz Date 31 Aug 73Telephone 305 371-5572 34
Area Code Number Extension

Checks payable to

Hapanicolaou Cancer Research Institute

Mailing address for check:

P.O. Box 6188Miami, Florida 33123

1003541916

-27-

Valentin Michael Yermakov, M.D.

Curriculum Vitae

- 3 -

MEDICAL SOCIETIES:

REDACTED

1003541979

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

Dogs will be housed in the animal facilities which are located on the ninth floor of the Research Building of Temple University Health Sciences Center. All dog experiments will be performed in the surgical research laboratory also located on the 9th floor of the same building. All laboratory determinations will be performed in the laboratory of the principal investigator which is located on the second floor of the hospital of the Temple University Health Sciences Center.

11. Additional facilities required:

None

1003541989

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Item # 10. Space and facilities available

tary needs. Adjacent to the animal room are 2 storage rooms approximately 5'x10' (50 sq. ft.) and 5'x13' (65 sq. ft.). These are used to store food, shavings and other sundry supplies. Also adjacent to the animal quarters is a behavioral study room 7'x12' (84 sq. ft.) used for O₂ consumption, locomotor activity and other studies when required. This room permits animals to be observed and studied in relative quiet. A separate room 10'x17' (170 sq. ft.) removed from the animal room by a corridor and 2 doors serves as office space and area for auditory stress studies. This separation prevents extraneous noise from bells, etc., to reach and disturb animals in the animal quarters. A washroom, approximately 12'x17' (204 sq. ft.) contains an automatic-spray washing machine and sinks which are used to sterilize and cleanse cages and water bottles. The main research laboratory approximately 16'x50' (800 sq. ft.) is provided with desks, table tops, cabinets and much of the equipment cited above. This room contains 3 water-sinks and is the area where autopsies, hematological, histological, and biochemical tests are performed and where calculations are done.

- b) Institute of Pathology
Downstate Medical Center, S. U. N. Y.
450 Clarkson Avenue
Brooklyn, N. Y.

At the Institute of Pathology, laboratory rooms and equipment are available for sectioning and automatic fixing and staining of the preparations and slides. They consist of microtomes, auto-technicons, microscopic equipment, glassware and accessory supplies. An electron microscope and fluorescent apparatus are available if these techniques are needed.

1003541971

Theodore A. Slotkin - Privileged Communication

can also be studied by continuous density gradient centrifugation, which provides a sensitive measure for evaluating small differences in the densities of different populations of vesicles (2, 4).

c. Secretion and recovery of amines and vesicles.

This can be evaluated using neurogenic secretion evoked by insulin-induced hypoglycemia or non-neurogenic amine loss produced by reserpine (3, 4).

Studies by this investigator utilizing these techniques have been published (1, 2, 3, 4). Experiments of this type were used to demonstrate the sequence of events during secretion and repletion of amine stores in normal adult rats, demonstrating that secretion of the vesicle contents is all-or-none, and that resynthesis of catecholamines is probably the rate-limiting step in repletion.

In addition, during the first half-year of this project, the techniques have been used to determine the maturational process in normal rats to serve as a base-line with which to compare SHR (see appended progress report) and to observe some of the alterations in the adrenal medulla of adult SHR. These studies suggest that altered neural input occurs in the SHR and that this may in turn slow maturation of the gland. Furthermore, determinations of the kinetic uptake constants suggest the possibility that the SHR may be somewhat more resistant to some antihypertensive agents (reserpine).

The first phase of the study - maturation in normal rats - has been completed, and preprints are appended describing the results in detail.

2. Previous work by other investigators: During prenatal and postnatal development, there is a marked increase in catecholamine levels in adrenergic neurons and in the adrenal medulla (5, 6, 7, 8), as well as changes in catecholamine synthesizing enzymes (5, 9). Although the necessary enzymes are present early in gestation (5), catecholamines do not appear until late in gestation, at a time when storage vesicles first become detectable (10, 11), suggesting that the storage vesicles play a determining role in the increase in adrenal amines. Consequently, the largest changes in catecholamine content occur in the postnatal period (0-6 weeks after birth). Spontaneously hypertensive Wistar rats (SHR) first show significantly elevated blood pressures towards the end of this period (5 weeks), along with disturbances in sympathetic catecholamine synthesis, storage and release (12). Similarly, in studies utilizing uninephrectomized rats treated with desoxycorticosterone acetate and NaCl, it has been shown that the resultant hypertension is accompanied by a defect in catecholamine storage such that cardiac storage vesicles become "leaky" (13). Westfall (14) has likewise demonstrated changes in catecholamine turnover in rats with elevated systolic blood pressures produced by chronic nicotine administration. Impairment of catecholamine storage has been implicated in essential hypertension in humans, as evidenced by increases in excretion of catecholamines and their metabolites in individuals with that disease (15).

In each case, hypertension was accompanied by a disturbance in sympathetic function probably involving impaired storage. Therefore, it is important to examine systematically the properties of the storage vesicles in at least one of the model systems. The SHR is probably the most reliable of all the models of hypertension to use for a study of this type: hypertension develops

1003541937

parameters which previously demonstrated differences will be measured. Since neural input will have been blocked, these differences will disappear if they were central (i.e. neural) in origin but not if the differences were inherent in the glands themselves.

Since preliminary studies suggest that altered sensitivity to reserpine and other uptake blockers (notably harmine) may occur in SHR, in vitro uptake studies will be conducted to determine sensitivity to these agents.

4. Significance: The sympathetic nervous system and its endocrine counterpart, the adrenal medulla, exert important regulatory functions on the entire cardiovascular system. During the first six weeks after birth, the adrenergic neurons and adrenal medullae of the rat undergo marked changes in catecholamine synthesis, uptake, storage and release. At the same time, hypertension begins to develop in spontaneously hypertensive Wistar rats which is associated with defects in the physiological disposition of sympathetic amines. The proposed study is designed to identify specific changes in the ability of the vesicles of the adrenal medulla to take up and store amines or to release them upon appropriate stimulation. The development studies could determine at what time after birth these changes occur. Only by direct measurement of the amounts of vesicular components and of vesicular properties can alterations of this type be identified. The developmental studies could also elucidate the nature of the process by which catecholamine stores increase during development and will indicate whether the rate-limiting step is the synthesis of vesicles, the synthesis of catecholamines, or the development of the ability of the vesicles to store the amines.

To summarize, the significance of the proposed study is that it may provide answers to the following basic problems:

a. Interaction of hypertension and the sympatho-adrenal system:

1. Is there an altered catecholamine turnover rate in hypertensive animals?
2. Is there an alteration in the ability to secrete amines upon stimulation?
3. What defect(s) is (are) responsible for alterations in (1) and (2), on the vesicular and subvesicular levels?
4. Do these alterations affect the sensitivity to antihypertensive agents?

b. Etiology of hypertension:

1. Do alterations in catecholamine stores precede the development of hypertension?
2. Can these alteration explain the hypertension?

c. Development of adrenal catecholamine stores:

1. What processes occur during the postnatal period to cause the increase in adrenal catecholamines?
2. Are these processes altered in hypertensive rats?

References

1. T.A. Slotkin, R.M. Ferris and N. Kirshner, Mol. Pharmacol. 7: 308 (1971).
2. T.A. Slotkin and N. Kirshner, Mol. Pharmacol. 7: 581 (1971).
3. T.A. Slotkin and N. Kirshner, Biochem. Pharmacol. 22: 205 (1973).
4. T.A. Slotkin and N. Kirshner, Mol. Pharmacol. 9: 105 (1973).

1003541940

8. Any additional facilities now required? Describe briefly: NO.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

NO.

1003541303

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

- a. Regan, T.J., and Moschos, C.B.: Effects of a chronic smoking program upon clotting and fibrinolysis in dogs. Presented at the Third Workshop Conference on Tobacco and Health, American Medical Association Education and Research Foundation, Newport Beach, Calif., 1972. (Abstract.)
- b. Ahmed, S., Levinson, G.E., Moschos, C.B., Oldewurtel, H., and Regan, T.J.: Effect of smoking nicotinized and nonnicotinized cigarettes on systolic time intervals. Clin. Res. 20: 359, 1972. (Abstract.)
- c. Ahmed, S.S., Moschos, C.B., and Regan, T.J.: Cardiovascular effects of chronic smoking in beagles. Circulation 45, 46: II-103, 1972. (Abstract.) Presented at the 45th Scientific Sessions, American Heart Association, Dallas, Texas, 1972.
- d. Ahmed, S.S., Moschos, C.B., Sethi, V., Ettinger, P.O., and Regan, T.J.: Comparative cardiovascular physiology of beagle and mongrel dogs. Physiologist 15: 69, 1972. (Abstract.) Presented at the 23rd Annual Fall Meeting, American Physiological Society, University Park, Pa., 1972.

12. Summary progress report (append in standard form as separate document, unless recently submitted)

investigations can show a precise localization of angiotensin I converting enzyme. Resolution of the order of 100Å should be achievable using either ferritin or peroxidase.

Type II alveolar cells

It is clear from our previous studies that further understanding of the genesis, maturation and release of lamellar bodies of type II alveolar cells will require major improvements in the preservation of phospholipid components during preparation of tissues for electron microscopy. We believe that these improvements will be of particular importance in understanding the effects of hormones and drugs, such as nicotine, on lamellar bodies and other organelles of type II cells.

Technical developments. One of the most striking features brought out by replicas of freeze-etched material is the extreme regularity of the leaflets in the lamellar bodies. Using several variations of standard dehydration procedures necessary for embedding, we, as others, have been unable to show the regular organization in thin sections. Indeed in most published pictures, lamellar bodies have looked like vacuoles containing some lamellae at their periphery. However, all available cytochemical and biochemical evidence indicates that the lamellar bodies and surfactant are predominantly lipid in nature. Sorokin (1967) suggested that the lamellar bodies are imperfectly preserved by conventional osmium-aldehyde fixation followed by processing in lipid solvents. An important phospholipid component of surfactant, dipalmitoyl lecithin, would clearly be vulnerable to conventional specimen preparation (Dermer, 1969).

Aqueous embedding media, avoiding alcoholic dehydration, should greatly enhance the preservation of lamellar bodies, however published protocols give inconsistent results and poor sectioning properties. A method suggested recently by Prof. R. Barnett (personal communication) incorporates a rational approach which we shall pursue first. In Barnett's method, tissue is fixed in glutaraldehyde (usually we use 2.5% glutaraldehyde in cacodylate buffer, pH 7.4). The polymerization reaction mixture is prepared, on ice, as follows: A solution of glutaraldehyde (50-75%) is mixed vigorously with pure carbohydrazide (m.p. 153°C, Eastman-Kodak or Polysciences) to give a final concentration of carbohydrazide of 3.3M. The preparation can then be frozen at -20°C or can be used immediately for embedding. When used for embedding, the glutaraldehyde-carbohydrazide solution is diluted serially to give solutions in water of 25%, 50%, 70%, 85%, 95% and 100%. Fixed tissues are moved through these serial dilutions until they have been exposed to the 100% solution. Then a few drops of the undiluted glutaraldehyde-carbohydrazide solutions are applied to dental wax, the tissue is added and polymerization begins at room temperature. After 8 hours at 37°C, the block can be applied to a chuck and processed from that point in the usual fashion.

In addition to preserving the regular organization of the leaflets of intracellular lamellar bodies, the aqueous embedding method should also preserve the organization of extracellular lamellar bodies and the airspace reticulum and surface film.

1003541918

Theodore A. Slotkin - Privileged Communication

Abstracts:

1. T.A. Slotkin and V. DiStefano, Urinary metabolites of harmine in the rat and their inhibition of monoamine oxidase. Fed. Proc. 28: 797, 1969.
2. T.A. Slotkin, V. DiStefano and W.Y.W. Au, Metabolism of harmine in rats and humans. Pharmacologist 11: 273, 1969.
3. T.A. Slotkin and V. DiStefano, A model of harmine metabolism in the rat. Fed. Proc. 29: 678, 1970.
4. T.A. Slotkin, Efflux of ^{14}C -epinephrine from bovine adrenal medullary granules. Fed. Proc. 30: 445, 1971.
5. T.A. Slotkin and N. Kirshner, Structure-activity relationships for uptake and storage of amines by isolated bovine adrenal medullary vesicles. Pharmacologist 13: 228, 1971.
6. T.A. Slotkin and N. Kirshner, Depletion of rat adrenal medullary constituents following insulin. Fed. Proc. 31: 521, 1972.
7. T.A. Slotkin, Uptake of epinephrine and metaraminol by isolated rat adrenal medullary vesicles following insulin administration. 5th Int. Cong. on Pharmacol. 217, 1972.
8. T.A. Slotkin, Uptake and storage of amines in isolated adrenal medullary vesicles of developing rats. Fed. Proc. 32: 783Abs, 1973.
9. T. A. Slotkin, Maturation of Adrenal Catecholamine Storage Vesicles of the Rat. Pharmacologist in press.

1003541945

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

July 19, 1973

Grant Application No. 923

To: To the committee comprising Drs. Bing, Gardner, and Jacobson

Subject: Guenther Boden, M.D., Temple University, Philadelphia
New application No. 923
"Effect of Nicotine and Cigarette Smoke on Secretin Secretion".

History

This proposal was Case No. 175, and application was encouraged.

Application No. 923 requests \$36,206 plus one additional year.

Documents Submitted

Attached is application dated 7/11/73 (10 pages).

A copy of the key publication listed as #1 on page 7 of the application has been provided and will be forwarded if you wish. We shall also be glad to obtain for you copies of any of the other publications cited.


F.W.N.

FWN:wg
Encl.

1003541985

4.

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

A. Stanley Weltman, Ph.D.

25

Valentin M. Yermakov, M.D.

10

Stefan Schwan, M.D.

25

REDACTED

Technical

Vijay Pandhi, M.S.

100

Leroy Johnson, B.S.

100

Ratilal Vaidya

50

Caretaker

30

Pathology Technician

100

REDACTED

Sub-Total for A

B. Consumable supplies (by major categories)

Wistar and Spontaneously Hypertensive Rats

1250

Feed and Bedding

1100

Glassware, Chemicals, Recording Physiograph

Paper, Linens, etc.

1350

Pathology-Technical stains, slides, chemicals, etc.

5000

8700

Sub-Total for B

C. Other expenses (itemize)

Publications

200

Sub-Total for C

200

Running Total of A + B + C

REDACTED

D. Permanent equipment (itemize)

Thin Layer Chromatography Apparatus

900

(Tanks; U.V. Lamp; Plates; etc.)

Flame Photometer (Coleman #21; NA and K)

750

Furnace, Ashing Oven and Temperature Control

650

Sub-Total for D

2300

E. Indirect costs (15% of A+B+C)

E

7316

Total request

REDACTED

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	\$9200	\$200		\$8010	REDACTED
Year 3						

1003541956

ok
10/7

Theodore A. Slotkin - Privileged Communication

8. Brief statement of working hypothesis: The development of hypertension is spontaneously hypertensive rats, in rats with surgically or pharmacologically induced hypertension, and in human essential hypertension, is associated with alterations in catecholamine storage. In spontaneously hypertensive rats, hypertension develops at a time when the catecholamine stores are undergoing marked development changes. It is proposed to study the process by which the stores increase during development in normotensive and hypertensive rats in order to define specific defects in sympatho-adrenal function.

9. Details of experimental design and procedures (append extra pages as necessary)

1. Previous work by applicant: The adrenal medulla is often utilized as a model of the sympathetic neuron; both tissues arise embryonically from the neural crest and both have the ability to synthesize, store and secrete catecholamines. Each contains storage vesicles which can accumulate amines by a mechanism which is stimulated by ATP - Mg^{2+} and blocked by reserpine. The vesicles contain dopamine beta-hydroxylase (DBO), chromogranins and adenine nucleotides as well as catecholamines; it is accepted generally that the catecholamines and adenine nucleotides (primarily ATP) form a storage complex in a molar ratio of 4 to 1.

For the past three years, the research of this investigator has been concerned with the development of sensitive and appropriate methods for the evaluation of the properties of the catecholamine storage vesicles of the adrenal medulla (1, 2, 3, 4). The adrenal medulla was chosen because it provides a more useful model than sympathetic nerves with which to study amine storage. Purified adrenal vesicles can be obtained in high yield by a relatively rapid discontinuous density gradient technique, while much lower yields of comparably purified sympathetic nerve vesicles are obtained after more lengthy procedures. Furthermore, the high concentration of storage vesicles in the adrenal permits the evaluation of properties which would be far more difficult to determine in nerve vesicles. Because of the development of these methods, the following parameters can be measured:

- a. Uptake and storage capabilities of the vesicles. The simultaneous measurement of the accumulation of radioactively labeled amines by the vesicles along with the efflux of endogenous and labeled amines permits evaluation of these two parameters. The rate of efflux is determined by the stability of storage, while the accumulation is a measure of storage stability and affinity for uptake. Additionally, the relative importance of ATP - Mg^{2+} stimulated uptake can be evaluated by measuring the accumulation of metaraminol, an amine which is incorporated by a primarily ATP - Mg^{2+} -independent mechanism (1, 2).
- b. Concentrations of intravesicular components.
Vesicles are purified by discontinuous sucrose density gradient centrifugation. The subcellular distributions of catecholamines, ATP and DBO can thus be readily determined, along with the fragility of the vesicles (see METHODS section). The buoyant density of the vesicles

1003541936

Summary Progress Report - March 16, 1972 - July 31, 1973.

The primary objectives of our research are

- 1) to examine the relationships of fine structure of pulmonary endothelial cells to the selective processing of circulating hormones, and
- 2) to examine the effects of hormones and drugs on the fine structure of type II alveolar cells and to study possible participation of type II alveolar cells in the processing of substances of the prostaglandin type.

Structure and function of pulmonary endothelial cells

As discussed in earlier progress reports, all available evidence supports the concept that bradykinin and angiotensin I, like the adenine nucleotides, are metabolized by enzymes on or close to the surface of pulmonary endothelial cells. Our hypothesis is based on the findings that angiotensin I and bradykinin disappear during a single circulation through the lungs (Ryan et al., 1970 and 1971). Disappearance is owing to enzymic degradation and not to tissue uptake nor transfer to extravascular spaces. Specific metabolites are recovered in nearly quantitative yields in the pulmonary venous effluent. Blood enzymes play little or no role as angiotensin I and bradykinin are degraded no less rapidly during circulation through lungs freed of blood.

Using bradykinin or angiotensin I labelled intrinsically with ^{14}C or ^3H , the apparent volumes of distribution and mean transit times of radioactivity do not exceed those of blue dextran ($\text{MW} > 2,000,000$), a compound unlikely to leave the intravascular space (Ryan et al., 1972). Furthermore, we have found that angiotensin I and bradykinin are degraded to characteristic products by the plasma membrane-caveolae intracellulares fraction of whole lung homogenates.

However, endothelial cells of the pulmonary capillaries are extremely thin (as little as 0.01μ) and because of this thinness and because of the variety of cell types within the lungs, it could be argued that rapid uptake or transfer of angiotensin, followed by rapid release of metabolites might be difficult to detect in our experiments. Therefore we decided to study the metabolism of angiotensin I by pure monolayers of endothelium collected from mainstem pulmonary artery.

We obtained Kutchen preparations of endothelium by applying strips of cellulose acetate paper to the luminal surface of the pulmonary artery. We have shown that cells obtained by this method are viable in short-term tissue culture. The coherence of the endothelium can be demonstrated by staining the paper and attached cells with methylene blue and cleaning in xylene. Electron microscopy presented a problem since the cellulose acetate paper is soluble in many of the preparative solvents. Nevertheless, by developing an agar sandwich technique (Smith and Ryan, 1973) we were able to examine the cells in the electron microscope and further to demonstrate that the endothelium occurred as a pure monolayer and was not contaminated by other cell types or extracellular material. The endothelial cells show a preferential attachment to the cellulose acetate paper and we were unable to separate them. However, we were able to set up the

1003541923

Embedding techniques which preserve lipids as they exist in vivo should have great impact on the remainder of the research proposal. Firstly, it will allow a firm baseline for the search, using thin sections, needed to confirm substructures revealed by freeze-etching. Secondly, since shadowing materials of unknown thickness are used in freeze-etch techniques, the most reliable measurements of substructural components and their spacing should be provided by examination of thin sections. Thirdly, freeze-fracture techniques use more tissue in a more random manner (one accepts the fracture wherever it occurs) than do standard techniques. Therefore, studies of well-preserved thin sections give more control—as will be required for serial sections, cytochemistry and immunocytochemistry.

We believe that cytochemistry will be a very valuable ancillary procedure for approaching a variety of problems concerning the origins, maturation and fate of lamellate bodies. For example, during its intracellular life, the lamellar body is known to contain a spectrum of enzymes including acid phosphatase, (Hatasu and Nakamura, 1965; Goldfischer et al., 1968; Mehan, 1972) alkaline phosphatase (Sorokin, 1967) and esterases (peptidase enzymes?) capable of hydrolyzing p-nitrophenylthiol esters (Vatter, et al., 1968). On the other hand, there is little or no information on what happens to these enzymes when the lamellar inclusion is expelled into the airspace. Possibly all enzymatic activity is lost. If this is so, the point should be established. However, if the enzymes survive in the airspace, it would be important to demonstrate their survival as there may be functions of expelled enzymes in terms of remodeling the surface lining or the reticulum or even in the processing of organic inhalants such as those which may be contained in tobacco smoke. Having developed methods of preserving the airspace lining, we propose to use standard methods for acid and alkaline phosphatase (Gomori, 1952) and the methods of Vatter et al., for esterase enzymes. The studies will be coordinated with investigations of enzymic activities of lung lavage fluid rendered acellular by differential centrifugation. Cell organelles and reticular substances will be purified by sucrose density gradient ultracentrifugation (Stein et al., 1969). Lipids of the supernatant will be examined by thin layer chromatography (Stein et al., 1969) and phospholipids will be measured in terms of chloroform-extractable phosphorus (Brown et al., 1964).

We further believe that selective staining techniques may bring information to bear on the mechanisms by which expelled lamellar bodies unfold. Drawing on studies of the expulsion of the contents of mucocysts of *Tetrahymena* (Satir et al., 1972), once fusion of the cyst with the plasma membrane has occurred, there is an immediate release of the highly-concentrated mucoid contents. Satir postulates that biological energy is not required. The entry of water followed by hydration of the mucoid would greatly expand the material, forcing it out of the stoma and into the surrounding media.

Possibly, the analogy applies to lamellar bodies, which are known to contain sialomucin (Luke and Spicer, 1965). We propose, using ruthenium red (a selective stain for acidic polysaccharides, Luft, 1964, 1965), to examine lamellar bodies within and without the giant alveolar cell for the disposition of acidic polysaccharides. Although the results would not, by themselves, be conclusive a disposition of acidic polysaccharides between the lipid leaflets should, on hydration in the airspace, cause the leaflets to spread. Furthermore, a hydrated

1003541919

REFERENCES

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28. Hadley, H. G.: Med. Rec. 153:267, 1941.
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1003541967

of his proposed research than he has outlined in the proposal, and I would hope that he does limit the scope of the proposed investigation and also clarify several of the issues presented within such a limited scope.

1003542004

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1003541969

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

a) The space and facilities available at the Laboratories for Therapeutic Research, Brooklyn College of Pharmacy are as follows:

The Laboratories were designed for the purpose of conducting animal investigations in physiology, pharmacology, endocrinology, biochemistry, and experimental therapeutics. It is a Laboratory which is equipped for work in all of these fields and possesses histological, microscopic, biochemical and animal-surgical equipment necessary for the conduct of detailed investigations.

The animal rooms are air-conditioned, the animals are housed in metal cages and an automatic cage-washing machine is available. The permanent equipment in addition to the cages includes: (1) Leitz Ortholux Binocular Microscope, (1) Sartorius Selecta Precision balance, (1) F.P.E. Precision balance, (2) ovens, (2) Incubators, (2) Refrigerators, (1) Freezer, (1) Turner Fluorometer, Model 110, (1) Coleman Spectrophotometer, Model 6, (1) Spectronic 20 (Bausch & Lomb), (1) Servall Centrifuge, (1) Adams Dynac Centrifuge, (1) Torbal Torsion Balance, (1) Bausch and Lomb freezing and (1) Spencer rotary paraffin microtome, a Technicon for processing histological specimens, (1) Beckman pH Meter, (2) A.H. Thomas shakers, (1) De-mineralizer Unit (Barnstead), (1) Corning AG-1 Glass Distilling Apparatus, (1) Hot plate, (1) Stir-Jack, (1) Elconap Constant Temperature Water Bath, (1) Friden Calculator, (1) Friden 130 Electronic Calculator, (1) Marchant Cogito 566 PR Calculator, (1) General Radio Oscillator, Type 1210 C and amplifier, (1) Audiogenic-Stress Belling Chamber, (1) Stainless Steel Pipette Washer and a miscellany of glassware and accessory equipment. (1) Narco-Biosystems, Desk Model DMP-4B, Physiograph and accessory equipment for systolic blood pressure measurements.

The animals are housed in an air-conditioned room approximately 22' x 27' (594 sq. ft.), provided with an exhaust system, which can contain 8-9 animal racks. Cages for mice or rats are available depending upon the particular study. The animal room contains water facilities and a drainage system for proper sanitation (see attached sheet page 19)

11. Additional facilities required:

12. Biographical sketches of investigator(s) and other professional personnel (append):

A.S. Weltman (pages 21-24), V.M. Yermakov (pages 25-27), S. Schwan (pages 28-29)

13. Publications. (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

1003541955

There have been many epidemiological studies which reveal a significant association between cigarette smoking (CS) and the morbidity and mortality from arteriosclerotic heart disease (ASHD). It is noteworthy that this relationship is less conspicuous than that for CS and diseases of the respiratory system. Further a causal role of CS to ASHD is less clear. Most antagonists to views relating CS to ASHD propose a common genetic factor which may be responsible for both. Support for this view appears from the outstanding "twin studies" of Lundman.¹ Some skepticism concerning the role of CS in ASHD has also been derived from the inconsistencies and somewhat paradoxical results obtained from epidemiological studies considering the duration of CS and events occurring in ex-smokers when compared to non-smoking populations. ^{2,3,4}

Results of pharmacologic investigations concerning the effect of CS or nicotine on the cardiovascular system often reveal divergent results which, at least in part, appear to be related to differences in dosage of nicotine utilized and experimental technics employed. Also, most of these studies might be regarded as acute and therefore unrevealing with respect to such a chronic disorder as ASHD. Nevertheless, there is evidence which indicates that the cardiovascular effects of CS are synonymous with that of nicotine.^{5,6,7} In man short term studies have disclosed a slight pressor effect, tachycardia and an increase in cardiac output following inhalation of cigarette smoke or intravenous injections of nicotine. ^{8,9,10} Although some ¹¹ have found a more prolonged pressor effect and tachycardia in chronic smokers than in non-smokers following CS others have denied such differences. ^{12,13} Studies of CS or nicotine in animals reveal comparable cardiovascular effects to those

1003541861

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1003541966

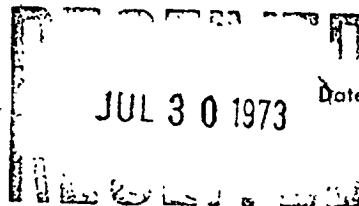
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CONTINUATION

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)



Date: July 24, 1973

1. Principal Investigator (give title and degrees):

Theodore Alan Slotkin, Ph.D., Assistant Professor of Pharmacology

2. Institution & address:

Dept. of Physiology and Pharmacology
Duke University
Durham, North Carolina 27710

3. Department(s) where research will be done or collaboration provided:

Dept. of Physiology and Pharmacology

4. Short title of study:

Maturation of the Adrenal Medulla: Catecholamine stores in normal and hypertensive rats.

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 1 year

7. Brief description of specific research aims: During the first weeks after birth, there are major changes in the catecholamine stores of the sympathetic neuron and its endocrine counterpart, the adrenal medulla. During the same period (5 weeks) spontaneously hypertensive Wistar rats (SHR) first show significantly elevated blood pressures. It has been reported that by the end of this period there is a change in catecholamine turnover in the SHR. It is proposed: (1) to study the maturation of adrenal catecholamine stores in SHR and normal rats, and (2) to elucidate the mechanisms by which changes in catecholamine turnover have occurred. Techniques developed by this investigator will be used to measure the number of storage vesicles, their amine uptake and storage capabilities, and the degree to which stores can be mobilized during stress or depleted by drugs. These will include measurements of the amounts of vesicular components and the ability of the vesicles to incorporate isotopically labeled catechol- and non-catecholamines. Specifically, this study will attempt to determine the sequence and rate-limiting step(s) in the development of adrenal amine stores and to evaluate differences between normals and SHR in the development of the ability to maintain or secrete the amines. By identifying specific defects or changes in amine storage, these data could provide insight into the etiology of hypertension and into the interaction between hypertension and sympathetic nervous system function.

1003541935

REFERENCES

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1. Konturek, S.J., Solomon, T., McCreight, W.G., Johnson, L.R. and Jacobson, E.D. Effects of nicotine on gastrointestinal secretions. *Gastroenterology* 60:1098-1105, 1971.
 2. Bynum, T.E., Solomon, T.E., Johnson, L.R. and Jacobson, E.D. Inhibition of pancreatic secretion in man by cigarette smoking. *GUT* 13:361-365, 1972.
 3. Doll, R., Jones, F.A. and Pygott, F. Effect of smoking on the production and maintenance of gastric and duodenal ulcers. *Lancet* 1:657-662, 1958.
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 9. Ulcer 'smoke ring' clarified. *Medical World News*, June 4, 1971. p. 23.
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1003541990

Proposed Research

Fine structural localization of the angiotensin I converting enzyme

As discussed in the summary progress report and in previous progress reports, all existing evidence indicates that angiotensin I and bradykinin are metabolized by enzymes on or close to the luminal surface of pulmonary endothelial cells. However, it has not proved possible to test the concept by any single existing technique. Previously, we showed that the plasma membrane fraction of whole lung homogenate does in fact convert angiotensin I to angiotensin II and degrades bradykinin to characteristic lower homologs (Ryan and Smith, 1971; Ryan et al., 1972). Although these data support the hypothesis, we do not know what proportion of the plasma membrane is derived from endothelial cells and what proportion comes from other cell-types. Therefore, we subsequently began efforts to isolate endothelial cells to obtain direct evidence of their metabolic capabilities (Ryan and Smith, 1973).

Results with isolated endothelial cells of the mainstem pulmonary artery are described below (section 12) in greater detail. Our results show that pure monolayers of endothelial cells can in fact convert angiotensin I to angiotensin II. While the ability of a pure line of endothelial cells to metabolize angiotensin I adds strength to our hypothesis on the subcellular site of the relevant enzymes, we believe that two further studies are required to round out definitive tests.

Firstly, in our studies of pulmonary endothelial cells isolated on cellulose acetate paper, we found means of carrying monolayers of cells in primary culture. Thus the possibility exists of obtaining endothelial cells in sufficient quantities, through scaled-up cell culture, to allow harvesting of pure plasma membrane fractions from a known cell line. Given sufficient starting material, we propose to use the method developed previously in this laboratory (Ryan and Smith, 1971) to isolate plasma membrane and to test the reactivities of the preparation with angiotensin I and bradykinin.

As a second approach, we have agreed to a collaborative study with Dr. Frederic Dorer, Cleveland Veterans Hospital, to raise specific antibodies to the converting enzyme of hog lung. Dorer and colleagues (1972) have succeeded in purifying the enzyme to homogeneity and we have begun the requisite immunizations. Although we project booster immunizations, we have already obtained an antibody reactive with angiotensin converting enzyme as demonstrated by the Ouchterlony technique. Further analysis is required, but evidence on hand indicates that the antibody is monospecific. The antibodies will be used in an attempt to establish the subcellular site of converting enzyme by cytoimmunologic techniques. For the purposes of electron microscopy, antibody will be labelled with ferritin in one series of experiments and with horse radish peroxidase in a second series. Ferritin is itself electron-dense after reaction with OsO_4 . The peroxidase, on reaction with diaminobenzidine, will yield an electron-dense product suitable for high magnification studies.

Judging from similar studies conducted by others for other purposes (Moriarty and Halmi, 1972), we believe it likely that the proposed cytoimmunologic

1003541917

CURRICULUM VITAE

Name: **REDACTED**

Date of Birth: **REDACTED**

Immigration Status: **REDACTED**

Marital Status: **REDACTED**

Educational Qualifications:

1. B. Sc. University of Mysore, India. May, 1964.
2. B. Pharm. Bangalore University, India. June, 1968.
3. Ph.D. Natural Product Chemistry. Philadelphia College of Pharmacy and Science. Philadelphia, May, 1973.

Professional Experience:

1. Graduate Assistant (1968-1973). Philadelphia College of Pharmacy and Science.

Publications:

1. Murthy, S.N.S. and A.D. Marderosian: Isolation and identification of the toxic saponin principles from sea cucumbers. Proc. 3rd Food and Drugs from Sea Conference. Rhode Island, 1973 (in press).
2. Murthy, S.N.S. and A.D. Marderosian: The isolation and identification of Rodiasine from *Ocotea venenosa*. Lloydia. 1973 (in press).
3. Marderosian, A.D. and S.N.S. Murthy: The analysis of old samples of *Canabis sativa* L. Proc. Soc. Econ. Bot., 1972 (Abstract).
4. Marderosian, A.D., H. Pinkley, F. Goldstein and S.N.S. Murthy: The ethanobotany, phytochemistry and pharmacology of Amazon arrow poison used by Kofan Indians. Proc. II International Botanical Congress, San Diego, 1969. p. 72.

1003541997

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29. Weltman, A.S., Sackler, A.M., Johnson, L. and O'Connor, G.: Aspects of Protein, Carbohydrate and Fat Metabolism in Whirler Mice. *Fed. Proc.* 29:778, 1970.
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31. Weltman, A.S., Sackler, A.M. and Schwartz, R.: Maternal Effects on Behavior and White Blood Cells of Isolated Female Mice. *Life Sciences* 9:291-300, 1970.
32. Weltman, A.S. and Sackler, A.M.: Metabolic and Endocrine Aspects of the Whirler Mutation in Male Mice. *Acta Endocrinologica* 64:347-358, 1970.
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1003541976

-28-

CURRICULUM VITAE

NAME:

DR. STEFAN SCHWAN

HOME ADDRESS:

REDACTED

PHONE:

SOCIAL SECURITY NUMBER:

MARITAL STATUS:

REDACTED

PLACE & DATE OF BIRTH:

REDACTED

PELIMINARY EDUCATION:

REDACTED GRAMMER SCHOOL
1940 - 1944REDACTED HIGH SCHOOL
1944 - 1945REDACTED HIGH SCHOOL
HIGH SCHOOL DIPLOMA - 1951

PROFESSIONAL EDUCATION:

Medical Faculty of the University of Gdansk
1951 - 1957 PhysicianFrom 1956 - 1966 Employed in the Department of
Pathology at the University in Gdansk as Assistant
Professor.

1962 - Received the scientific degree of Medical Doctor.

1966 - Received the degree of a specialist in Pathology.

TRAINING:

Oncological Department of the Polish Scientific
Academy - 1960 - 1964.Fellowship in the Department of Pathology in the
Mary Curie-Skłodowska Oncological Institute in
Warsaw - 1963 - 1965.Since 1967 - Worked in the Department of Pathology
in the State Hospital in Gdansk and in the Childrens
Surgical Clinic of the University in Gdansk.From 1970 to 1971 - Worked as a ships surgeon in the
Polish Ocean Lines.1971 - Arrived in West Germany and worked as a
Chief of the Department of Pathology and Cytology in
the Institute of Microbiology and Clinical Chemistry
in Weingarten.Arrived in the United States on December 15, 1971
on a permanent visa.

1003541980

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Laboratories for Therapeutic Research Research Institute of The Brooklyn College of Pharmacy, is a non-profit basic research institution at Brooklyn College of Pharmacy. Costs of Plant Operation are jointly shared by the College and the Laboratories. Expenses for the Laboratories Operation are from private contributions.			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

1003541957

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name A. Stanley Weltman, Ph.DSignature A. Stanley Weltman Date 7/16/73Telephone 212 MA 2-1790
Area Code Number Extension

Checks payable to

Brooklyn College of Pharmacy

Mailing address for checks

600 Lafayette Ave., Brooklyn, N.Y. 11216

Responsible officer of institution

Typed Name Seymour SchertzTitle ComptrollerSignature Seymour Schertz Date 7/16/73Telephone 212-MA2-4040
Area Code Number Extension

(Bargmann and Knoop, 1956). In terms of origin and in terms of the progressive maturation of lamellar bodies up until the time of their release from the cell, it is important to determine the spatial and possible structural relationships to other organelles. Using the complexity of the substructural array seen in freeze-etching as an index of maturity, it may be possible to determine the stages in the intracellular processing of surfactant material and to document contributions from other organelles. The sequence of events in the synthesis, through subsequent release of zymogen (from rough ER through Golgi apparatus to exocytosis) from the exocrine pancreas (Palade and Siekevitz, 1956) could be taken as an example of such a life history.

The possible origins of lamellar bodies from mitochondria has been debated for many years. Although we see no clear conclusions, some reviewers (Scarpelli, 1968) have pointed out that mitochondria and lamellar bodies have different histochemical reactivities and have taken this as conclusive evidence that lamellar bodies could not arise from mitochondria. In the light of more recent evidence (Werb and Cohn, 1972) on the formation of phagolysosomes, structures derived from plasma membrane and lysosomes, it is evident that histochemical reactions may be quite different at late stages of evolution from those of contributing structures.

We believe it to be unlikely that the instances we have found of mitochondria and lamellar bodies enveloped by a single membrane are fortuitous. Myelin figures, which in thin section have some resemblance to lamellar bodies, are known to be formed by mitochondria under certain adverse conditions (see for e.g., Tuchweber et al., 1972). However, the envelopment of lamellar bodies and mitochondria by a single membrane can be interpreted in a variety of different ways. The broadest interpretation is that communications occur between these organelles, and the communication may or may not be the consequence of one organelle evolving to the next. Taking previous cytochemical studies into account with our findings, we regard it as important to make a systematic search for communications of lamellar bodies with other organelles, most prominently multivesicular bodies (Vatter et al., 1968) and lysosomes (Hatasa and Nakamura, 1965). In this manner we intend to provide further information on the assembly of materials ultimately expelled into the airspace.

Having located transitional forms in the search just described, we will use selective cytochemical techniques [succinic dehydrogenase for mitochondria, (Ogawa and Barrnett, 1965) acid phosphatase for lysosomes, (Essner and Novikoff, 1961) etc.] to determine which enzymes lamellar bodies lose or gain during their intracellular life cycle. These studies will be coordinated with the investigation described above for examining enzymes of the airspace reticulum.

Effects of hormones. Studies by Redding et al. (1972) and Olsen (1972) indicate that thyroid and some neurohumoral agents can profoundly influence the synthesis and secretion of lamellar bodies. If thyroid hormone does in fact stimulate synthesis and assembly, cells under the influence of thyroid hormone should show more transitional forms, a point that would greatly facilitate our studies on the maturation of lamellar bodies. Similarly, examination of fetal lung may provide the same opportunity. In addition, the effects of isoproterenol on secretion will facilitate examination of mechanisms of delivery of the lamellar body to the cell membrane. There is the further point, of far greater importance, that the effects of these hormones could possibly have therapeutic implications.

1003541921

The following publications of the principal investigator are relevant to the proposed research:

1. Boden, G. and Chey, W.Y.: Preparation and specificity of antiserum to synthetic secretin and its use in a radioimmunoassay (RIA). *Endocrinology* 92:1617-1624, 1973.
2. Boden, G., Dinoso, V. and Owen, O.E.: Immunological comparison of natural and synthetic secretins. *Horm. Metab. Res.* 1973 (August).
3. Boden, G.: The secretin radioimmunoassay. Chapter 27 in "Methods of Hormone Radioimmunoassay. B.M. Jaffe and H. Behrman, eds. Academic Press. New York and London. 1974 (in press).
4. Gulati, S.C. and Boden, G.: Organ distribution and excretion of radioactivity after injection of ^{125}I -secretin in rats. Presented at the Eastern Section APCR, Boston, January, 1973. *Clin. Res.* 20:869, 1972.
5. Boden, G., Gulati, S.C. and Essa, H.: Influence of intraduodenal HCl on immunoreactive secretin (IRS) levels in dogs. Presented at the National Meeting APCR, Atlantic City, April, 1973. *Clin. Res.* 21:508, 1973.
6. Boden, G. and Dinoso, V.P.: Immunoreactivities of natural and synthetic secretins. Presented at the 74th Annual Meeting of the American Gastroenterological Association, May 1973, New York, N.Y. *Gastroenterology* 64:878, 1973.
7. Boden, G., Gulati, S.C., Owen, O.E. and Shuman, C.R.: The insulinogenic effect of endogenous secretin. Presented at the 33rd Annual Meeting of the American Diabetes Association, Chicago, June, 1973. *Diabetes* 22: 304, 1973 (Suppl. 1).
8. Boden, G., Gulati, S.C., Essa, N. and Owen, O.E.: Influence of endogenous secretin on insulin secretion. To be presented at the 18th Congress of the International Diabetes Federation, Brussels, Belgium. July, 1973.

1003541994

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

June 7, 1973

Grant Application No. 905

To: The committee comprising Drs. Gardner, Jacobson and Meier
Subject: Edward F. Domino, M.D., University of Michigan, Ann Arbor
New application No. 905
"Neuropsychopharmacological Effects of Chronic Nicotine"

History

This investigator was supported by CTR from January 1, 1959 through December 31, 1972.

Application No. 873, requesting continuation from 1973, was denied by the SAB.

Application No. 905 requests \$52,885, plus two additional years in lesser amounts.

Documents Submitted (attached)

1. Application dated March 20, 1973.
2. C.V.'s of Dr. Domino and Mr. Spaulding (graduate student).
3. Final report on Dr. Domino's previous CTR grant, dated August 6, 1972 through December 31, 1972.
4. Reprints of the publications listed on page 3a of the application have been provided, and will be forwarded to you if you so request.

Comment

Attached are copies of opinions from Neal E. Miller, Walter B. Essmann, and Murray E. Jarvik.

FWN:gh


F. W. N.

1003541939

REFERENCES

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83. Manual of Fluorimetric Clinical Procedures, G. K. Turner Assoc. 1966 (2/62). Procedure for Total Serum Cholesterol.
84. Goss, J. E. and Lein, A.: Clin. Chem. 13:36, 1967.
85. Manual of Worthington Biochemical Corp. Procedure for serum total triglycerides and free glycerol.
86. Zilversmit, D. B. and Davis, A. K.: J. Lab. Clin. Med. 35:155, 1950.
87. Vernikos-Danellis, J., Anderson, E. and Trigg, L.: Endocrinol. 79:624, 1966.
88. Vochten, R. F. C., Hoste, J., Delaunois, A. L. and DeSchaepe-dryver, A. F.: Analytica Chem. Acta. 40:443, 1968.
89. Snedecor, G. W.: Statistical Methods, Iowa State College Press, Ames 1949.
90. Sokabe, H.: Nature 205:90, 1965.
91. Kolletsky, S., Shook, P. and Rivera-Velez, J.: Proc. Soc. Exp. Biol. Med. 134:1187, 1970.
92. Kolletsky, S., Shook, P. and Rivera, Velez, J.: in "Spontaneous Hypertension: It's Pathogenesis and Complications," edited by Okamoto, K., Springer-Verlag, N. Y., 1972, page 199.
93. Ogino, K., Kira, J. and Matsunago, M.: in "Spontaneous Hypertension: It's Pathogenesis and Complications," edited by Okamoto, K., Springer-Verlag, N. Y., 1972, page 210.

1003541970

PHARMACOLOGY

1003541983

#836AR1 -- ESSMAN

1003542022

Item # 13. Publications

For a more complete list of some of the Laboratories 61 publications, see Item #12.

Metabolic and Endocrine Effects of Lysergic Acid Diethylamide (LSD-25) on Male Rats, A. S. Weltman and A. M. Sackler, J. Endocrin. 34:81-90, 1966.

Effects of Levels of Audiogenic-Seizure Susceptibility on Endocrine Function of Rats, A. S. Weltman, A. M. Sackler and H. Owens, Physiology and Behavior 3:281-284, 1968.

Mescaline Hydrochloride Effects on the Endocrine Activity of Male Albino Mice, A. Stanley Weltman, Arthur M. Sackler and Ralph Schwartz, Experimental Medicine and Surgery 26: No. 4, December 1968.

Pre-maternal Isolation Effects on Behaviour and Endocrine Function of Offspring, A. M. Sackler, A. S. Weltman, R. Schwartz and P. Steinglass, Acta Endocrinologica 62:367-384, 1969.

Metabolism Rate, Biochemical and Endocrine Alterations in Male Whirler Mice, A. S. Weltman, A. M. Sackler, A. S. Lewis and L. Johnson, Physiology and Behavior 5:17-22, 1970.

Metabolic and Endocrine Aspects of the Whirler Mutation in Male Mice, A. S. Weltman and A. M. Sackler, Acta Endocrinologica 64:347-358, 1970.

Effect of Mescaline HCl on Resistance of Male Mice to Histamine Stress, A. S. Weltman, A. M. Sackler and L. Johnson, Journal of Pharmaceutical Sciences 59: 1659-1661, 1970.

Plasma Protein and Free Fatty Acid Levels in Male Whirler Mice, A. S. Weltman, A. M. Sackler and G. O'Connor, Experientia 27:878, 1971.

Nicotine Effects in Spontaneously Hypertensive Rats (SHR), A. S. Weltman, V. Pandhi, S.D. Kraus and L. Johnson, Fed. Proc. 32: No. 3, 806, March 1973.

1003541972

13. Publications

Olds, M.E. and Domino, E.F.: Comparison of muscarinic and nicotinic cholinergic agonists on self-stimulation behavior. J. Pharmacol. Ex. Ther. 166: 189-204, 1969.

Domino, E.F. and VonBaumgarten, A.M.: Tobacco cigarette smoking and patellar reflex depression. Psychiat. Digest 30: 50, 1969.

Domino, E.F.: A role of the central nervous system in the cardiovascular actions of nicotine. Arch. Int. Pharmacodyn. Ther. 179: 167-179, 1969.

Stitzer, M., Morrison, J., and Domino, E.F.: Effects of nicotine on fixed interval behavior and their modification by cholinergic antagonists. J. Pharmacol. Exp. Ther. 171: 166-177, 1970.

Domino, E.F.: Neuropsychopharmacology of nicotine and tobacco smoking. Chapt. 2, pp. 5-31, In Smoking Behavior: Motives and Incentives, W.L. Dunn, Jr., Ed., V.H. Winston and Sons, Inc., Washington, D.C., 1973.

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16. Other sources of financial support

List financial support from all sources, including own institution, for this and related research projects

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Adrenal catecholamine fluxes in SHR	N.C. Heart Ass. 1972-73 A-10	2,500	1-15-73 -- 1-14-74
Catecholamine stores in SHR	Duke University Inman Fund	3,750	7-1-73 -- 6-30-74

PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Amine stores of developing normal and hypertensive rats.	American Heart Ass.	50,000	requested for 9-1-73 -- 8-31-76

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made"

Principal investigator

Typed Name Theodore Alan Slotkin, Ph.D.

Signature Theodore Slotkin Date 7/23/73

Telephone 919-684-5224
Area Code Number Extension

Checks payable to

Duke University

Mailing address for checks

C. B. Huestis, V. P. Business & Finance
203 Allen Building
Duke University
Durham, North Carolina 27705

Responsible officer of institution

Typed Name William G. Anlyan, M.D.

Title Vice President for Health Affairs

Signature W. G. Anlyan Date 7/27/73

Telephone 919-684-5125
Area Code Number Extension

1003541950

Essman, W.B.: Chapt. 4. Nicotine-related neurochemical changes: Some implications for motivational mechanisms and differences. pp. 51-65 In Smoking Behavior: Motives and Incentives, W.L. Dunn, Jr., Ed., V.H. Winston and Sons, Inc., Washington, D.C., 1973.

Holmstedt, B. and Lundgren, G.: Arecoline, nicotine and related compounds, tremorgenic activity and effect on brain acetylcholine. *Ann. N.Y. Acad. Sci.* 142: 126-142, 1967.

Morrison, C.F. and Armitage, A.K.: Effects of nicotine upon the free operant behavior of rats and spontaneous motor activity of mice. *Ann. N.Y. Acad. Sci.* 142: 268-276, 1967.

Nelson, J.M. and Goldstein, L.: Improvement of performance on an attention task with chronic nicotine treatment in rats. *Psychopharmacologia (Berl.)* 26: 347-360, 1972.

Olds, M.E. and Olds, J.: Approach-avoidance analysis of rat diencephalon. *J. Comp. Neurol.* 120: 259-295, 1963.

Rosecrans, J.A.: Brain area nicotine levels in male and female rats with different levels of spontaneous activity. *Neuropharmacology* 11: 863-870, 1972.

Szilagyi, P.I.A., Green, J.P., Brown, O.M., and Margolis, S.: The measurement of nanogram amounts of acetylcholine in tissues by pyrolysis gas chromatography. *J. Neurochem.* 19: 2555-2566, 1972.

Tenen, S.S.: An automated one-way avoidance box for the rat. *Psychonomic Sci.* 6: 407-408, 1966.

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

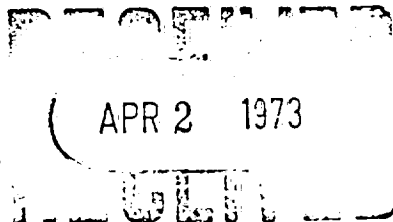
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: March 20, 1973

1. Principal Investigator (give title and degrees):

Edward F. Domino, M.D., Professor of Pharmacology



2. Institution & address:

University of Michigan
Ann Arbor, Michigan 48104

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Neuropsychopharmacological Effects of Chronic Nicotine

5. Proposed starting date: July 1, 1973

6. Estimated time to complete: Three year study with the first year's research to be completed
June 30, 1974

7. Brief description of specific research aims:

1. To determine if the behavioral effects of nicotine tartrate given several times per day change on chronic administration in the rat.
2. To correlate the content of brain nicotine with its behavioral effects especially if on chronic administration tolerance is observed.
3. To determine the effects of chronic nicotine administration on locomotor activity in the mouse.
4. To determine the effects of nicotine on brain acetylcholine content in the rat under the same nicotine treatment schedule as in 1 and 2.
5. To determine the effects of chronic nicotine administration on neocortical and limbic system activation in the cat.

1003542005

14. First year budget:

A. Salaries (give names or state "to be recruited")	% time	Amount	Fringe Benefits		
Professional (give % time of investigator(s) even if no salary requested)					
Guenther Boden, M.D.	25				
Shreekan Murthy, Ph.D.	100				
			REDACTED		
Technical					
To be recruited	100				
			REDACTED		
			REDACTED		
Sub-Total for A					
B. Consumable supplies (by major categories)					
Glassware		500			
Dogs		3,000			
Supplies for animal surgery		1,000			
Supplies for radioimmunoassay:					
Radioisotopes		1,500			
Reagents and antisera		500			
		6,500			
Sub-Total for B					
C. Other expenses (itemize)					
Service contract for gamma counter		680			
		680			
Sub-Total for C					
Running Total of A + B + C			REDACTED		
D. Permanent equipment (itemize)					
Desk calculator		250			
Centrifuge (International Model HN-S)		410			
		660			
Sub Total for D					
E Indirect costs (15% of A+B+C)		4,636			
Total request			REDACTED		
15. Estimated future requirements:					
Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	500	680	4,955	REDACTED
Year 3					

OK
R.e.H

1003541991

Item #9. Details of Experimental Design and Procedures

Balance to the nearest 0.1 mg. Histological preparations will be made of the heart, aorta, pulmonary artery, renal blood vessels, mesenteric blood vessels, testes, liver, lungs, brain, eye, pancreas, and pituitary to determine the extent and presence of either associated cardiovascular pathologies, hormonal functions and possible effects of nicotine administration in the SHR and NR groups.

Based on gross and microscopic observations, the animals will be examined for cardiac infarctions (scarring and hypertrophy), periarteritis nodosa, nephrosclerosis, cerebral hemorrhage, lung involvement, etc.

Depending upon specific requirements, tissues and organs will be fixed in 10% formalin or Bouin's fixative and stained after sectioning with hematoxylin and eosin or corresponding appropriate stains (i.e. van Gieson's stain, elastic-van Gieson's stain, PAS stain and elastic-PAS stain, fat stains, etc.).

All blood pressure, biochemical, organ weight, etc. data will be analyzed for statistical significance by standard t test and variance procedures (89) whenever appropriate. Correlation procedures (89) will be used to analyze i.e., cholesterol and blood pressure values, etc., to determine the direct or inverse relationships of the various biochemical parameters with hypertension or nicotine administration. The laboratory has available a Cogito 566 PR model calculator (Marchant) as well as a Friden 130 Electronic Calculator for computation of the data. In addition, the laboratories has available, the facilities of the Long Island University, Brooklyn Center, Computer Center. The Computer Center has an IBM Model No. 1130-3C computer and accessories available for the statistical analyses.

Thus, this detailed biochemical, endocrine, histological and pathological series of investigations should aid in determining short and long range effects of nicotine on physiological and hormonal processes of spontaneously hypertensive and normotensive rats. The investigation should aid in clarifying present inconsistencies and ambiguities concerning nicotine in relation to blood pressure, blood lipid profile, etc., and their pathological implications. This study should possibly resolve questions concerning harmful, neutral or beneficial aspects of nicotine intake to man and perhaps yield information pertinent to essential hypertension.

1003541965

Theodore A. Slotkin - Privileged Communication

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3. T.A. Slotkin and V. DiStefano, A model of harmine metabolism in the rat. J. Pharmacol. Exp. Ther. 174: 456-462, 1970.
4. T.A. Slotkin and V. DiStefano, Cardiovascular and respiratory effects of harmine. Proc. Soc. Exp. Biol. Med. 133: 662-664, 1970.
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7. T.A. Slotkin and N. Kirshner, All-or-none secretion of adrenal medullary storage vesicle contents in the rat. Biochem. Pharmacol. 22: 205-219, 1973.
8. T.A. Slotkin and N. Kirshner, Recovery of rat adrenal amine stores after insulin administration. Mol. Pharmacol. 9: 105-116, 1973.
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14. T.A. Slotkin, Reserpine, in "Neurotoxins: Their Pathophysiological Actions" vol. 2 (L.L. Simpson and D.R. Curtis, eds.) in press.
15. N. Kirshner and T.A. Slotkin, Secretion and recovery of catecholamines of the Adrenal Medulla. Biochem. Pharmacol. in press.
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1003541944

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

July 20, 1973

Grant Application No. 833A

To: The Committee comprising Drs. Bing, Jacobson, and Sommers

Subject: A. Stanley Weltman, Ph.D., Brooklyn College of Pharmacy, N.Y.
Continuation application No. 833A (no commitment).
"Effects of Nicotine in Spontaneously Hypertensive and
Normotensive Rats"

History

Our initial grants to Weltman were for 1972 and 1973, the latter as terminal. Therefore the enclosed request competes as a new application.

Application No. 833A requests \$58,386 plus one additional year. The current level of support is \$42,663 per year.

Documents Submitted (attached)

1. Application dated 7-17-73 (29 pages).
2. Semi-annual Progress Report No. 3, January 1 to June 30, 1973.
3. Abstract, 1973 Federation Proceedings "Nicotine Effects in Spontaneously Hypertensive Rats". We have the full manuscript of this paper, now pending with American Heart Journal; copies will be forwarded if you wish.

Comment

The work proposed for 1973 apparently will not be finished. Dr. Weltman states that although the pathology collaboration recently established promises to be satisfactory, the pathologists at Downstate have not been keeping up with tissues sent to them.

Experimental design has now been improved by replacing nicotine administration in drinking water, with subcutaneous injection in glycerol-gelatine vehicle for slow absorption (except weekends when nicotine is given in drinking water).

Besides completing the unfinished work, Dr. Weltman wishes to add triglycerides and phospholipids to the determinations of plasma lipids.

F.W.N.
F.W.N.

FWN:wg
Encls.

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THE ROCKEFELLER UNIVERSITY

NEW YORK, N.Y. 10021

May 9, 1973

Dr. Frederic W. Nordsiek
The Council for Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York, N. Y. 10022

Dear Dr. Nordsiek:

The project "Neuropsychopharmacological Effects of Chronic Nicotine" submitted by Edward F. Domino proposes to test the effects of chronic administration of nicotine on three behavioral tasks with rats and one with mice. These are reasonably representative samples of behavioral tasks out of a much larger population of such tasks. No rationale is given for the selection of these particular tasks and evidence is cited for acute effects of nicotine on only one of them, locomotor activity in the mouse.


It also proposes to study the effect of nicotine on brain acetylcholine content and utilization in the rat and, presumably, to correlate the conditions that produce such an effect, if any is found, with the conditions producing behavioral effects, if any, in the three rat tasks, not the mouse one in which an acute effect had been observed. No special rationale for selecting the acetylcholine effect is presented, except of course that nicotinic effects can be produced by acetylcholine so that some feedback inhibition on synthesis could occur.

Finally, it proposes to do an experiment on the shift of EEG activation from the reticular formation to the hippocampus without giving any special rationale for this study.

While the acute-chronic variable is a significant one and there is some reason for each of these studies, the project as a whole certainly is a heterogeneous collection and was not very impressive to me. Perhaps someone more familiar with the literature on nicotine would find justifications which are not specifically presented in the proposal. It probably is worthwhile supporting, provided that you have lots of money or if you know that this is an excellent man.

The other project (from Thomas C. Westfall) has been evaluated by Dr. Larissa Pohorecky, a bright Assistant Professor of Pharmacology in my laboratory. She agrees with my evaluation of the Domino project and believes that the one by Westfall is much better.

Sincerely,


Neal E. Miller
Professor

NEM:emg

1003542001

REFERENCES

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1003541968

Item #7. Brief Description of Specific Research Aims

and normotensive rats as related to age and prolonged nicotine administration. Histological preparations and examinations of the heart, aorta, pulmonary artery, renal blood vessels, mesenteric blood vessels, brain, lungs, fundus of the eye, lungs, kidneys, testes, liver, spleen, thymus, pituitary and pancreas have and will be used to determine the extent of associated cardiovascular pathology and endocrine involvements in the respective organs. The frequency and extent of cardiac infarctions (scarring and hypertrophy), periarteritis nodosa, nephrosclerosis, cerebral and lung pathologies will be carefully assayed. Thus, this intensive biochemical, endocrine and histological study associated with repeated measurements of systolic blood pressure and body growth should aid in determining the degree of pathological involvements and nicotine-related effects in spontaneously hypertensive and normotensive rats. This investigation should aid in clarifying present day inconsistencies and ambiguities concerning possible harmful, neutral or beneficial influence of nicotine intake to man and its possible involvement with essential hypertension. Of added import, the recent findings of significant decreases in total cholesterol levels in 6 and 29 week studies (oral, 2.28 mg/kg/day of nicotine alkaloid) in the spontaneously hypertensive test rats merit further investigation regarding the effects of nicotine on the blood lipid profile.

Various investigators have reported no apparent increased activity in the renin-angiotensin system of the SHR strain (90, 91). Additional research have likewise indicated no evidence that the renal humoral pressor system of the SHR was hypereactive (92) or that renin was increased in the SHR (76). Others have reported that the SHR seem to be hyperresponsive to the hypertensive-inducing effects of rat kidney extracts (93). During the investigation, procedures will be attempted to assess the possible effect of nicotine on the renin-angiotensin system of the spontaneously hypertensive and normotensive strains.

The habit of smoking tobacco has long been suspected and accused of being an etiological factor leading to cardiovascular diseases (hypertension, arteriosclerosis, atherosclerosis, etc.) (1-11). Epidemiological and statistical studies have claimed a greatly increased risk of coronary heart disease, morbidity, and mortality from cardiovascular disease in smokers than in non-smokers (12, 13).

In recent years (14) a strain of spontaneously hypertensive rats (SHR) has been selectively bred, which various investigators consider to be most appropriate for studies relative to essential hypertension (14-16). Various extirpative, as well as exogenous hormone procedures have been used to demonstrate and test the active role of the pituitary-adrenal axis and pituitary-thyroidal role in inducing and maintaining the hypertensive state in the SHR strain (17, 18). Other investigations have demonstrated the contributing role of the adrenal medullary activity and catecholamine output with the development of the spontaneously hypertensive state (19-21). Evaluation of pathological changes in blood vessels, heart, kidneys, brains, etc. of the spontaneously hypertensive rats (22) have paralleled changes found in cardiovascular diseases and essential hypertension in man.

Considerable epidemiological and pathological studies have been devoted to determine effects and association of tobacco smoking to emphysema, chronic bronchitis, cardiovascular diseases and lung cancer (4, 7). It has been cited statistically that heavy smokers have higher mortality rates from coronary heart disease than non-smokers (4). Whether the habit of smoking tobacco can be related to the development of hypertension and coronary diseases has, thus, long been the subject of much discussion (4, 7)

1003541958

Theodore A. Slotkin - Privileged Communication

rapidly and requires no pharmacological or surgical intervention, the animals are available as a genetically pure strain, and inbred normotensive Wistar rats provide a valid control (12). It is of additional interest that, although the mechanism of hypertension may be different from SHR, essential hypertension in humans is probably genetic in origin (16).

Utilizing the techniques developed by this investigator, this study will attempt to elucidate possible differences in the catecholamine stores of SHR and normal rats by examining the uptake, storage and release of amines from adrenal medullary vesicles during the period from birth until the development of hypertension.

3. Methods: Litters of SHR and normotensive Wistar rats will be sacrificed at intervals of several days over the period from birth until the development of hypertension in the SHR group (about 6 weeks) (12). The adrenal glands will be removed and analyzed as follows:

a. Determination of the number and contents of storage vesicles. The adrenal glands will be homogenized in isotonic sucrose, centrifuged at 800 x g for ten minutes, and the supernatant will be layered on 1.6 M sucrose and centrifuged for 2 hours at 140,000 x g. The latter centrifugation separates storage vesicles from most mitochondrial and lysosomal contaminants (17) as well as from broken vesicle membranes (3). All fractions will be assayed for catecholamines (CA) (trihydroxyindole method, 18), for ATP (firefly method, 19) and for dopamine beta-hydroxylase (DBO) (periodate oxidation method, 20). DBO is an enzyme associated with both the soluble and membrane-bound fractions of the storage vesicles (21), and the determination of DBO activity therefore provides an estimate of the number of storage vesicles present. The low levels of DBO present at the early stages of development may require the pooling of glands from several animals to obtain sufficient enzyme activity. The ratio of vesicular catecholamines to DBO provides a measure of the sequence of amines and vesicles: if CA/DBO remains constant during development, this would suggest that the rate-limiting step in development is vesicle synthesis. If CA/DBO increases, then vesicle synthesis is not rate-limiting. Thus, alterations in DBO levels in developing SHR may indicate changes in the number of storage vesicles present, while alterations in CA/DBO may indicate a change in the limiting step in age-dependent CA increases.

ATP is an integral part of the catecholamine storage complex. If CA/ATP is less than the adult ratio of 4 during the period of development, this would imply that nucleotide accumulation is not rate-limiting in establishment of amine stores. If CA/ATP is constant throughout development, then nucleotide accumulation may be rate-limiting. In developing hypertensive rats, alterations in ATP levels may indicate impaired storage capabilities.

The fraction of vesicles ruptured during homogenization is fairly constant from preparation to preparation (3, 4). Therefore, the ratio of DBO in the broken vesicle membrane fraction to the DBO in the intact vesicle fraction may provide a measure of "fragility" of the storage vesicles in both normotensive and hypertensive rats.

Alterations in any of the above factors--number of vesicles, ATP levels, vesicle fragility--could alter catecholamine storage.

1003541938

as percent of successful avoidances. In addition, the mean percent escape and no responses will be calculated. A dose-effect curve to acute nicotine and after 2 weeks of chronic nicotine will be determined. A similar experimental design will be used for performance of this avoidance behavior. Animals will be trained to a 95% criterion of avoidance in sessions of 50 per day for a minimum of 200 trials.

b. Multiple FR, FI behavior.

Operant behavioral techniques for food reinforcement using the adult male albino Holtzman rat will be utilized. Animals will be maintained at 80% of body weight by food restriction and water at liberty. The experimental subjects will be shaped to press a lever for food pellets on a continuous reinforcement schedule. Once this behavior is learned they will be placed on a progressive schedule (FR₁₀, FI₆₀) until performance is stable. Stability criteria will be overall session rate (60 minutes) and session quarter life on the FI schedule. Animals will be run 5 days per week. No drugs will be administered on Monday or Tuesday. On Wednesday 0.9% NaCl and/or sodium tartrate controls will be given with nicotine on Friday. Once a dose effect curve for nicotine is obtained, the animals will be given the compound 3 times/day for a minimum of 2 weeks, 7 days per week to determine its behavioral effects on chronic administration. Only one dose of nicotine will be studied per day (a.m.). The other doses of nicotine will be given on the usual tid schedule at 12:00 noon and 4:00 p.m.

c. Self-stimulation behavior.

Adult male Holtzman rats will be operated under pentobarbital anesthesia. Bipolar twisted enamel insulated stainless-steel electrodes will be placed in the lateral hypothalamus as per Olds and Olds (1963). After several days of recovery the animals will be trained to press a lever on a continuous reinforcement schedule to obtain a brief electric shock 0.25 seconds duration, 60 Hz with a current ranging from 40-60 μ A. Animals will be given 5 daily sessions per week. A drug schedule similar to that described above will be used to determine the effects of acute vs. chronic nicotine.

d. Other behavioral endpoints.

Depending on the approved duration of this grant proposal and the results obtained, other behavioral endpoints including Sidman avoidance and awake-sleep cycle will be studied. Details of the procedure will not be given. Basically, a comparison of the acute effects in nicotine naive animals to those given nicotine chronically will be made.

2. Correlation of nicotine brain content with behavioral effects in rats.

If the above experiments (a through d) show striking differences in either tolerance or sensitization following chronic nicotine administration,

1003542007

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1003541996

Society for Experimental Biology and Medicine - accepted 1962
American College of Neuropsychopharmacology - Charter Fellow - 1962
American College of Clinical Pharmacology and Therapeutics -
Charter Member - 1963
National Association on Standard Medical Vocabulary - Consultant
Member - 1963
American Electroencephalographic Society - Associate Member -
1963 to 1966, Full Member - 1966 to present
Collegium Internationale Neuro-Psychopharmacologicum - accepted 1966
University of Michigan Research Club - 1971
Society for Neurosciences - accepted 1969
Japanese Pharmacology Society - accepted 1972

Professional Society Positions

Sigma Xi - Councilor - 1961 to 1963
American EEG Society - Chairman, Symposium on "Neurotransmitters,
Brain Activity and Relation to EEG" - Society Meetings, June 1967
American College of Neuropsychopharmacology - Chairman, Symposium on
"Antipsychotic Drugs" Society Meetings, December, 1967

Visiting Lecturer in Neuropsychopharmacology

Sinai Hospital, Detroit, Michigan
Ypsilanti State Hospital, Ypsilanti, Michigan
Wayne County General Hospital, Wayne, Michigan

1003542018

8. Any additional facilities now required? Describe briefly:

NONE

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Mr. Eliahu Heldman has been replaced by Mrs. Barbara Kornreich as a Grant Researcher on this project.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

See attached list.

1003542025

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

A neuropsychopharmacological laboratory is available under the principal investigator in Medical Science Building I, Rooms 6440, 7422 and 7447.

11. Additional facilities required:

As listed on p. 4, Item D, Permanent Equipment, behavioral programming and motor activity equipment is needed for the behavioral studies. A rat stereotaxic is needed for the self-stimulation experiments. A peak integrator is needed for a gas chromatograph. The gas chromatograph itself is already available.

12. Biographical sketches of investigator(s) and other professional personnel (append):

Edward F. Domino

Theodore C. Spaulding

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See page 3a

Source: <https://www.industrydocuments.ucsf.edu/docs/gvym0000>

1003542011

REDACTED

p. 12

Theodore A. Slotkin - Privileged Communication
CURRICULUM VITAE

Hannah O. Green

Date of Birth: October 14, 1935

Place of Birth:

REDACTED

Marital Status:

Education:

1953-1957	Carnegie Mellon University	B.S. Chemistry
1958-1964	Cornell University	M.S. Biochemistry
		Ph.D. Biochemistry

Professional Experience:

6/57 - 6/58	Chemist, Jones and Laughlin Steel Corporation Research Laboratory, Pittsburgh, Pa.
9/64 - 12/66	Research Associate, Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii.
2/69 - 12/69	Research Associate, Department of Physiology and Pharmacology, Duke University Medical Center, Durham, N.C.
8/70 - ^{9/71} present	Research Associate, Department of Biochemistry, Duke University Medical Center, Durham, N.C.

Publications:

Oppenheimer*, H. L., J. Mercouroff, and G. P. Hess, Biochim. Biophys. Acta, 71, 78 (1963). "Characterization of the Difference Spectrum of Diisopropylphosphoryl- α -chymotrypsin versus α -Chymotrypsin. IV. The Environment of Tryptophyl Residues."

Oppenheimer, H. L., and G. P. Hess, Nature, 198, 689 (1963). "Difference Spectrum of Diisopropylphosphoryl-trypsin versus Trypsin."

Labouesse, B., H. L. Oppenheimer, and G. P. Hess, Biochem. Biophys. Res. Comm., 14, 318 (1964). "Conformational Changes Accompanying the Formation of Chymotrypsin-substrate Complexes. Evidence for the Involvement of an N-Terminal α -Amino Group in the Activity and the Conformation of the Enzyme."

* Maiden name

1003541946

CURRICULUM VITAE

Theodore C. Spaulding

Born: June 20, 1946

Home Address: 16-C Yum Yum Apartments
Carrboro, North Carolina 27510
Phone: (919) 942-1897

Present Position: Graduate Student
Department of Pharmacology
School of Medicine
University of North Carolina
Chapel Hill, North Carolina 27514
Phone: (919) 966-1151

Education:

1964 - 1969 B.S., Duquesne University (Pharmacy)
1969 - University of North Carolina (Pharmacology; Advisor:
Dr. William L. Dewey) (Thesis Research - Pharmacology
of Phenitrone, a reported antagonist of marihuana)

Experience:

1969 - 1970 Graduate Teaching Assistant, School of Pharmacy,
Dispensing Laboratory
1970 - 1971 Graduate Teaching Assistant, Pharmacology Laboratory
1971 - Graduate Teaching Assistant, Pharmacology Lectures
and Pharmacology Laboratory. Lecturer in Pharmacology,
School of Nursing

Fellowships:

1970 - Fellow of the American Foundation for Pharmaceutical
Education

Honorary Societies: Rho Chi

Scientific Societies: American Association for the Advancement of Science
American Pharmaceutical Association

Research Interests:

Drugs which affect the central nervous system and their interactions
with neurochemical transmitter systems.

1003542020

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Secretin secretion in normal subjects and in patients with diabetes mellitus	Grant in aide, Pfizer Pharmaceutical Co., N.Y. N.Y.	\$15,000	2/73 - 2/74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Influence of endogenous secretin on insulin regulation	AM 16348 01 PHS - NIH Pending	39,491	

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Guenther Boden, M.D.

Signature G. Boden Date 7/9/73

Telephone 215 221 3089
Area Code Number Extension

Responsible officer of institution

Typed Name Mr. David W. Siegel

Title Associate Vice President for Administration

Signature D. W. Siegel Date 7/11/73

Telephone 215 221 3246
Area Code Number Extension

Checks payable to

to University Health Sciences Center

Mailing address for checks

attn: Mr. David W. Siegel
Temple University Health Sciences Center
400 N. Broad Street
Philadelphia, Pa. 19140

1003541992

FROM WALTER B. ESSMAN, M.D., Ph.D.
Re Grant Application #905
May 21, 1973

RESEARCH PROPOSAL EVALUATION

Dr. Edward F. Domino

Neuropsychopharmacological Effects of Chronic Nicotine

The behavioral studies proposed for this investigation are not impressive and raise several questions regarding their over-all significance to the tissue studies proposed in the later section of the proposal. It is further noted that the behavioral techniques utilized have little relationship to one another, so that it is difficult, if not impossible, to make statements about shuttle-box avoidance that might be relatable in any respect to positively reinforced operant responses, electric self-stimulation or waking sleep cycles. It would be more appropriate, perhaps, to design such experiments using quantitatively low, high activity labeled nicotine, in conjunction with cold-loading doses, so that the time course of behavioral changes can be more meaningfully related to what now seems to be relatively independent experiments in the second portion of the proposal. The same comment might be made with regard to Experiment 3, which might, perhaps, be more appropriately be undertaken as the initial experiment in this proposal.

Insofar as Experiment 4 is concerned, there are a number of methodological problems which might be pointed to. Microwave irradiation as a method of sacrificing mice or for achieving rapid fixation of tissue is a highly unsatisfactory technique when applied to a consideration of the cholinergic system. Its efficiency is by no means comparable to that of Near-freeze methods, nor does it provide for a very satisfactory procedure if discrete tissue morphology

8. Brief statement of working hypothesis:

2.

Since the present investigation encompasses manifold aspects, the proposal primarily intended to determine the relationship and possible mechanisms via which nicotine may induce hypertension and/or hypotension during acute and/or prolonged administration of the drug. Thus, the investigation attempted and intends to explore alterations in various hormonal titers and biochemical parameters to determine the role of nicotine and hormones on blood pressure levels as well as certain aspects of carbohydrate, fat and salt metabolism and regulation, etc. Thus, biochemical (adrenal catecholamine and corticosterone and plasma corticosterone, glucose, cholesterol, FFA, total protein, and Na^+ and K^+ levels and urinary 17-ketosteroids) as well as organ weights and histological preparations will be measured to ascertain adrenomedullary, glucocorticoid, mineralocorticoid and perhaps gonadal (urinary 17-ketosteroid) role on blood pressure regulation due to nicotine.

To date, testing of male spontaneously hypertensive and normotensive rats (Wistar strains) in an unanesthetized state with 2.28 mg/kg of nicotine alkaloid per day have not revealed a biphasic effect as reported by Wenzel et al (31,32) with anesthetized female Sprague-Dawley rats (normotensive). Since Wenzel et al (32) reported hypotensive effects with higher doses in the normotensive Sprague-Dawley rats, the question arises whether male Wistar rats are more susceptible to equivalent (see attached sheet p.11)

9. Details of experimental design and procedures (append extra pages as necessary)

Five week old immature male rats of the spontaneously hypertensive strain (SHR) developed by Okamoto and Aoki (14) and normal (NR) Wistar rats (Carworth, Inc.) will be obtained from appropriate breeding laboratories. Hypertension is usually observable at 2 months of age in the SHR (20). Upon arrival all animals will be weighed on a Torbal Balance and carefully examined for signs of physical disability and ill-health. All rats will be housed in plastic cages (9"x11"x15") in groups of 4 rats per cage and weighed at weekly intervals. The animals will be permitted to acclimate for a 1 week period and will be supplied with Purina Lab Chow for food and permitted to drink water ad libitum. To determine the progressive development of spontaneous hypertension in the SHR rats, systolic blood pressure will be measured in the SHR groups as well as the normotensive rats (NR) at 6, 9, and 11 week age periods. The indirect tail-cuff method using the Narco-Biosystems Physiograph (Desk Model DMP-4B) will be used on unanesthetized rats for the systolic blood pressure measurements. Each rat will be prewarmed in an incubator for 15 minutes at 35 C prior to transfer to a Narco-Biosystems rat holder-warming unit (37 C).

At the completion of the 3 preliminary blood pressure readings, rats of each of the spontaneously hypertensive and normotensive groups will be matched according to systolic blood pressure and body weight for separation into appropriate test and control SHR and NR groups (4 groups).

Commencing at 11 weeks of age after determination of base-line systolic blood pressures, nicotine alkaloid (Eastman Kodak) will be administered subcutaneously, twice daily at 9:00 A.M. and 4:00 P.M. The dose will be divided to ensure that the total dosage approximates 2.28 mg/kg/day. This has been calculated to be equivalent to 2 packs of cigarettes/day. On week-ends, oral administration procedures will be used by supplying drinking water containing appropriate doses of nicotine alkaloid based on water consumption measurements. When injected subcutaneously, the nicotine will be administered in the form of a slow absorption and releasing aqueous vehicle by dissolving the appropriate nicotine concentrations in a sterile 2% glycerin - 2% gelatin preparation. Control spontaneously hypertensive and normotensive rats will receive corresponding injections of the 2% glycerin - 2% gelatin preparations.

1003541954

Teaching and Research Interests

Teaching. Application of audio and visual aids to medical education. New methods and philosophy of integrated teaching of basic and clinical material involving the nervous system at predoctoral and postdoctoral levels.

Research. Neuro- and psychopharmacology as a means of understanding brain function in animals and man.

A. Specific research problems.

1. Cholinergic neurotransmitters and interactions with psychoactive drugs.
2. Drugs affecting levels of consciousness (wakefulness, coma, psychotomimetic states, sleep and dreaming).
3. Neuropsychopharmacology of nicotine and smoking.
4. Biological and pharmacological alterations in schizophrenia.

B. Specific techniques

1. Neuropharmacologic
 - a. Electrophysiologic: evoked potentials, EEG, EMG
 - b. Computer analysis (Analog Applied Dynamics, TMC-CAT, IBM 1800)
2. Psychopharmacologic
 - a. Conditioned avoidance behavior
 - b. Self-stimulation behavior
 - c. Operant behavior
3. Radiochemical
 - a. C^{14} labeled precursors of neurotransmitter substances
 - b. O_2 burning technique and analysis
 - c. Scintillation counting
4. Neurochemical
 - a. ACh
 - b. AChE, ChAc, ChE, DMT, and NMT
 - c. 5-HT, 5-HIAA, bufotenine and related indole alkylamines

1003542019

Theodore A. Slotkin - Privileged Communication

Publications (continued)

Labouesse, B., K. Carlsson, H. L. Oppenheimer, and G. P. Hess
in "Structure and Activity of Enzymes" (Goodwin, T. W.,
J. I. Harris, and B. S. Hartley, editors), Academic Press,
New York, 1964, p. 71. "Characterization of a Residue
Controlling the Activity and Conformation of Chymotrypsin."

Oppenheimer, H. L., B. Labouesse, K. Carlsson, and G. P. Hess,
Federation Proceedings, 23, 315 (1964). "Role of N-Terminal
Isoleucyl Group in Conformation and Activity of Chymotrypsin."

Oppenheimer, H. L., B. Labouesse, and G. P. Hess, J. Biol.
Chem., 241, 2720 (1966). "Implication of an Ionizing Group
in the Control of Conformation and Activity of Chymotrypsin."

Oppenheimer, H. L., and R. H. McKay, Federation Proceedings,
25, 585 (1966). "Function of Zinc in Horse Liver Alcohol
Dehydrogenase."

Oppenheimer, H. L., R. W. Green, and R. H. McKay, Arch. Biochem.
Biophys., 119, 552 (1967). "Function of Zinc in Horse
Liver Alcohol Dehydrogenase."

Green, H. O., J. Moritz, and L. Lack, Biochim. Biophys. Acta,
231, 550 (1971). "Binding of Sodium Taurocholate by
Bovine Serum Albumin."

Green, H. O. and J. A. Reynolds, Federation Proceedings, 30,
1065 (1971). "Protein Components of Porcine Brain Myelin."

References:

Dr. George P. Hess
Department of Biochemistry
Cornell University
Ithaca, New York 14850

Dr. Robert H. McKay
Department of Biochemistry and Biophysics
University of Hawaii
Honolulu, Hawaii 96822

Dr. Leon Lack
Department of Physiology and Pharmacology
Duke University Medical Center
Durham, North Carolina 27706

Dr. Jacqueline A. Reynolds
Department of Biochemistry
Duke University Medical Center
Durham, North Carolina 27706

1003541947

REPRINTED FROM FEDERATION PROCEEDINGS
MARCH 1973, VOL. 32, NO. 3, PART 1 OF TWO
PRINTED IN U.S.A.

NICOTINE EFFECTS IN SPONTANEOUSLY HYPERTENSIVE RATS (SHR).
A.S. Weltman, V. Pandhi*, S.D. Kraus and L. Johnson*. Labs.
Therapeutic Res., Brooklyn Col. of Pharmacy, Long Island Univ.,
Brooklyn, N.Y. 11216

The effects of nicotine in mature male SHR after a single s.c. dose and after 6 wks of oral intake were noted. In the acute study, rats were sacrificed 30 min. after injections of 0.5 or 1.0 mg/kg of nicotine or saline. The 1.0 mg/kg dose caused significant increases in plasma corticosterone. Significant depletions were found in adrenal corticosterone and epinephrine along with elevations in plasma FFA. The 0.5 mg/kg dose caused smaller, non-significant changes. No significant changes in plasma Na, K or cholesterol levels were found with either dose. In the subacute study, test rats were given 2.2 mg/kg of nicotine orally in water per day for 6 wks. This represents a "two-pack-a-day" dose of nicotine. A transient increase in systolic b.p. taken at 24 hrs. was significant. Increases observed after the 1st and 2nd wks. were not significant, nor were decreases at the 4th and 6th wks. During the first 5 wks., the test rats had significantly lower body wts. than controls but the differences became gradually less. At the 6th wk, there was no significant difference between the 2 groups. At sacrifice, plasma cholesterol levels were significantly lower in the test rats but there were no significant differences in other biochemical analyses or organ wts (liver, thymus, adrenals, testes, kidneys, heart, etc.). By the 6th wk, the test rats appeared to accommodate to nicotine. (Supported in part by CTR Grant 833)

1003541982

CURRICULUM VITAEName: Guenther Boden, M.D.Born: **REDACTED**Marital Status: **REDACTED**Children: **REDACTED**MEDICAL TRAINING:

<u>Institution and Location</u>	<u>Degree</u>	<u>Year Conferred</u>
Heidelberg University, School of Medicine	M.S.	8/27/56
Munich University, School of Medicine	M.D.	12/5/59
Intern - City Hospital and University Hospital, Hamburg and Berlin (Germany)		1/1/60-3/31/62
Resident - City Hospital for Dermatology Stuttgart, Germany		4/1/62-12/31/62
Assistant in Biochemistry - Tuebingen University, Dept. of Biochemistry, Tuebingen, Germany		1/1/63-4/30/65
Research Fellow in Medicine - E.P. Joslin Research Laboratory, Boston, Mass. (Dept. of Medicine, Harvard Medical School)		5/1/65-9/30/67
Assistant in Medicine - Peter Bent Brigham Hospital, Boston, Mass.		7/1/65-6/30/66
Assistant Resident in Medicine - Rochester General Hospital, Rochester, New York		10/1/67-9/30/68
Associate Resident in Medicine - Rochester General Hospital, Rochester, New York		10/1/68-2/28/70

APPOINTMENTS - MEDICAL SCHOOL:

Assistant Professor of Medicine and Assistant Director, General Clinical Research Center, Temple University Health Sciences Center, Temple University, Philadelphia, Pa.

3/1/70 -

Specialty Affiliations: **REDACTED**Licensure: Pennsylvania **REDACTED**Honors: Rochester, New York Regional Diabetes Award, 1970

1003541993

is to be retained. With regard to the latter, the investigator does not specify which region or regions of the brain he proposes to re-examine in rodents or whether his study has been designed for whole brain assessment of cholinergic changes for nicotine treatment.

The investigator does not clearly point out that the investigations of Homestead and Lundgren were carried out in rats, not mice, utilized whole brain assays and were treated with physostigmine salicylate, in order to preclude enzymatic hydrolysis. The total acetylcholine levels measured in this study were only obtained within the first 10 minutes following the injection of an inordinately large dose of nicotine barbiturate and it might be pointed out that there is no data available for times beyond 10 minutes.

The studies reported by Essman were carried out in the mouse and referred only to the tissue of the cerebral cortex which were measured 45 minutes following administration of nicotine sulphate, following which, changes in several storage pools of this amine were measured. A comparison of the different results from these two sets of experiments hardly seem to generate any area of controversy through which definitive further studies are going to settle any issue.

The final experiment proposed in section 5 seems completely unrelated to those studies which are outlined in previous sections of the proposal, and there is no indication of what significance these would have.

I certainly believe that the investigator is a highly competent, established researcher, but the proposal is in general disappointing, in that I find very little that would essentially contribute either new methodology or empirical data for behavioral, biochemical or electrophysiological research of nicotine pharmacology. I think that the author probably has more to say about the scope

1. Essman, W.B. Neurochemical changes in ECS and ECT. Seminars in Psychiatry, 1972, 4, 67-70.
2. Essman, W.B. Drug effects and learning and memory processes. In: Garattini, S., and Shore, P. (Eds.). Advances in Pharmacology and Chemotherapy. N.Y.: 8, 1971, Academic Press, Pp. 241-330.
3. Essman, W.B. Nicotine-related neurochemical changes: some implications for motivational mechanisms and differences. In: Dunn, W.J., (Ed.). Smoking Behavior, 1973, 281-283.
4. Essman, W.B. Effects of ECS on cerebral protein synthesis. In: Fink, M., Kety, S.S., McGaugh, J., and Williams, T. (Eds.). The Psychobiology of ECT. Washington, D.C., V.H. Winston & Sons, 1973.
5. Essman, W.B. Regional alterations of synaptic O-phosphorylethanolamine in differentially housed mice. Rass. Clin. Scient., 1973.
6. Essman, W.B. Effetti dell'elettroshock sulla neurochimica del sistema nervoso centrale, I. Rass. Clin. Scient., 1972, 48: 361-370.
7. Essman, W.B. Effetti dell'elettroshock sulla neurochimica del sistema nervoso centrale, II. Rass. Clin. Scient., 1973, 49: 5-23.
8. Essman, W.B. Tissue distribution and central effects of digoxin in mice: effects of an acute and chronic stress. Pharm. Res. Commun., 1973 (In Press).

1003542028

Item #7. Brief Description of Specific Research Aims

In doses absorbed by cigarette smokers during and shortly after smoking, nicotine has been found to increase heart rate, raise arterial pressure, dilate arterial blood vessels of muscles, while contracting those of the skin, increase cardiac output (9) and reduce the skin temperature of the extremities (23). Nicotine, thus, produces a complex array of cardiovascular responses and hemodynamic effects in which the precise mechanisms cannot be readily defined (24). In considering the pharmacological actions of nicotine, low doses stimulate the sympathetic ganglia, aortic and carotid chemoreceptors and catecholamine release from the adrenal medulla which can cause increased blood pressure and heart rate changes (25). Large doses block ganglionic transmission. In addition, nicotine also stimulates ganglia of the parasympathetic system and the pulmonary and coronary arterial receptors which induce lowering in blood pressure and heart rate values (25).

It is evident from smoking studies in man (5, 23) and animals (26, 27) that acute tobacco smoke and/or nicotine produce transient increases in blood pressure, etc. In epidemiological studies of tobacco smoking effects, Hadley (28) reported that the average blood pressure of smokers was somewhat less than non-smokers. Hammond and Horn (29) and Damon (7) were unable to establish a relation between cigarette smoking and hypertension. Blackburn, et al. (1) also reported lower distinct tendencies of systolic and diastolic blood pressure in chronic smokers but found higher basal pulse rates and resting pulse rates in smokers. Smoking has also been reported to cause larger rises in blood pressures of hypertensive subjects than in normal subjects (3).

Chronic studies with animals involving effects of nicotine on blood pressure have also been inconsistent. In part, these inconsistencies may be related to differences in species, strain, sex, dose, mode of administration, blood pressure procedures, etc. Haag et al. (26) exposing rats to chronic cigarette smoke for 2 years reported that tobacco smoke did not produce significant differences in blood pressure, evidence of hypertension but reported tendencies of lower blood pressure values towards the end of the study. In contrast, rabbits administered nicotine alkaloid in drinking water revealed significant and cumulative increases in systolic blood pressure from 0 - 24 weeks (30). However, with female rats Wenzel et al. (31) reported that chronic oral administration for 55 weeks with a nicotine dose of 2.28 mg/kg/day equivalent to 2 packs of cigarettes per day exerted a biphasic effect on blood pressure. Initially systolic blood pressure readings of anesthetized rats showed gradual increases up to 20 weeks followed by subsequent depressor effects on blood pressure upon continued nicotine administration. Larger oral doses (3.44 and 4.56 mg/kg/day), however, induced only depressor or hypotensive effects on the systolic blood pressure levels (32). Administration of either the "low" or "high" oral doses of nicotine alkaloid to renal hypertensive rats lowered systolic blood pressures to below control levels once renal hypertension was established (32). Bhagat (33) administering nicotine subcutaneously for 6 weeks and Westfall (34) for 8 weeks to rats reported gradual and significant increases in systolic blood pressures.

1003541959

the content of brain nicotine will be correlated with the behavioral changes. Brain nicotine will be determined using ^{14}C -labelled nicotine given under the same conditions as described above. Animals will be isolated to reduce radiochemical contamination. The regional distribution of ^{14}C -nicotine in the neocortex, basal ganglia, thalamus, hypothalamus, brainstem and cerebellum will be determined in acute vs. chronic nicotine treated animals as described by Rosecrans (1972). Animals will be sacrificed by decapitation, regional brain dissections performed and tissue homogenized in 0.1 N NaOH. Nicotine will be extracted into heptane containing 1.5% isoamyl alcohol. ^{14}C -Nicotine will be reextracted into 0.1 N HCl and counted in a scintillation counter.

3. Effects of chronic nicotine on locomotor activity in the mouse.

Morrison and Armitage (1967) have reported that single doses of nicotine from 0.1 to 0.8 mg/kg given subcutaneously progressively decrease spontaneous motor activity for a 60 minute period. In contrast, *d*-amphetamine, cocaine and caffeine cause a marked increase in activity. We propose to study the effects of chronic nicotine on mouse motor activity. Logarithmic doses of nicotine tartrate will be given intraperitoneally to establish a dose effect curve in nicotine naive and chronic nicotine treated animals. Swiss Webster adult male mice will be used. They will be placed on a 7:00 a.m. to 7:00 p.m. light and 7:00 p.m. to 7:00 a.m. dark cycle prior to use. The photo-activity unit of Motron-Produtor will be used to measure motor activity in the daytime. Saline treated controls will be used. The dose-effect relations of acute nicotine treated animals will be established. Then groups of six mice per dose of nicotine will be given the drug 5 times per day for 2 weeks. This dose will probably be in the vicinity of .32 mg/kg but will be selected on the basis of initial dose-effect data. After 2 weeks of chronic nicotine treatment, the dose-effect relations of nicotine will be determined. It is hypothesized that on chronic administration "stimulant" effects of nicotine will be observed.

4. Effects of nicotine on brain acetylcholine content and utilization.

Holmstedt and Lundgren (1967) reported a slight increase in mouse brain acetylcholine after nicotine. Inasmuch as the number of animals studied was small, they questioned this effect although the findings were just barely significant statistically ($P < .05$). In contrast, Essman (1973) reported that nicotine caused an approximately 50% decrease in total brain acetylcholine in the mouse with a marked change in the ratio of its bound, vesicular and free pools. We propose to reexamine some of these findings in both the mouse and in the rat using logarithmic doses of nicotine that relate to the behavioral data above. Animals will be sacrificed by microwave irradiation, their brains removed and assayed for acetylcholine using the gas chromatographic method of Szilagy *et al.* (1972). Time of sacrifice will be at time of peak nicotine effect, as determined from the behavioral studies. In addition, acetylcholine utilization will be measured using the technique of blocking acetylcholine synthesis with hemicholinium-3 and/or acetylseco-hemicholinium and measuring the rate of brain acetylcholine fall following nicotine. This technique has been used for other psychoactive drugs, as described by Domino and Wilson (1972).

1003542008

turnover of these molecules may well provide extremely sensitive indices of how stress and nicotine treatment interact, and the nature by which the individual or interactive contributions of both provide meaningful hypotheses concerned with the development of pathology related to processes within such cellular or organ systems, as well as being descriptive of the more basic mechanisms responsive to such biological agents.

Brief Statement of Working Hypothesis:

The functional basis upon which the proposed research project rests is the assumption that cellular mechanisms as models for the effects of acute and chronic stress will serve as a meaningful basis for assessing the interaction of such stress responses and treatment with nicotine by experimental means. In view of the strong evidence for the prevention, antagonism, or reordering of site-specific mitochondrial metabolism produced by stress, through inhalation of gaseous phases from nicotine treatment (Riesen & Kyle, 1969) both the feasibility of an organelle model system for stress - nicotine treatment, deriving from such sources as liver and lung tissue, and the feasibility of investigating other cellular and subcellular systems, have been strongly indicated. It is not the purpose of this research to explore the multi-faceted systems within which stress operates or nicotine treatment exerts measurable effects, but to focus primarily upon five tissue systems from which the cellular and subcellular biochemical data may be derived in terms of those molecules which appear to be most significant for the regulation of physiological and pathophysiological processes within such organ systems. Those general hypotheses which are generated both on the basis of research findings from our laboratory, as well as from other work relating to the primary goal of this project, are that (1) models of acute and chronic stress, respectively may be developed and may reflect, in responses observed for several organ systems, changes

1003542030

American Medical Association - Certificate of Merit - 1964, Section on Anesthesiology. Scientific Exhibit "Measurement of Visual Input During Various Levels of Consciousness in Man" with Drs. Corssen and Sweet

Consultantships

AMA Council of Drugs - 1957, 1959, 1963-67
 JAMA - Questions and Answers - 1962-67
 Neuro- and Psychopharmacology - Lafayette Clinic (State Psychiatric Research Hospital) Detroit, Michigan - 1959 to present
 Expert witness for the government on the Benzodiazepines, Librium and Valium. Depression and Stimulant Drugs, Food and Drug Administration Hearings, Oct. 26 and 27, 1966, Washington, D.C., Docket No. FDA-DAC-2, p. 4542-4801.
 Consultant to Panel on Neurological Drugs, Drug Efficacy Study, National Research Council, National Academy of Sciences, 1967-68

Editorial Positions

Member of Editorial Board, Journal of Pharmacology and Experimental Therapeutics, 1958 to 1965
 Member of Editorial Board, Journal of Neuropharmacology, Jan. 1962 to present
 Member of Editorial Board, Univ. of Michigan Med. Journal, Jan. 1964 to present
 Member, Advisory Board, Psychopharmacologia, 1967 to present
 Consulting Editor, Psychophysiology, 1968 to present

International Societies and Meetings

Participant - Fourth International Symposium on Tobacco Alkaloids and Related Compounds, Wenner-Gren Center, Stockholm, Sweden, February 1964
 Member - XXIII International Congress of Physiological Sciences, Tokyo, Japan, September, 1965
 Participant - International Collegium Neuro-psychopharmacologicum, March, 1966, Washington, D.C.
 Participant - International Collegium Neuro-psychopharmacologicum, April, 1968, Tarragona, Spain
 Invited Lecturer - Fourth Latin American Congress on Pharmacology, Caracas, Venezuela, July, 1972

NIH Study Sections

Member - Study Section on Pharmacology and Chemistry, National Institutes of Mental Health - 1965 to 1968

Professional Society Memberships

AAAS - accepted 1951, Elected Fellow, 1963
 Sigma Xi - elected 1952
 Central EEG Society - accepted 1952
 American Society for Pharmacology and Experimental Therapeutics - elected 1953
 New York Academy of Sciences - accepted 1953
 Washtenaw County Medical Society - accepted 1958

1003542017

#642C-GOLDSTEIN

1003542051

905

UNIVERSITY OF CALIFORNIA, LOS ANGELES

BERKELEY • DAVIS • IRVINE • LOS ANGELES • RIVERSIDE • SAN DIEGO • SAN FRANCISCO



SANTA BARBARA • SANTA CRUZ

DEPARTMENT OF PSYCHIATRY
SCHOOL OF MEDICINE
THE CENTER FOR THE HEALTH SCIENCES
LOS ANGELES, CALIFORNIA 90024

4 June 1973

Dr. Frederic W. Nordsiek
Associate Scientific Director
The Council for Tobacco Research -- U.S.A., Inc.
110 East 59th Street
New York City, New York 10022

Dear Dr. Nordsiek:

Re: Your grant application #905

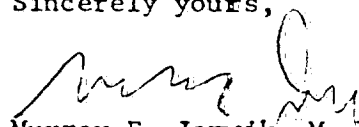
I trust that the following comments will be helpful in your determination concerning the proposed study of "Neuropsychopharmacological Effects of Chronic Nicotine":

The study of the effects of chronic nicotine is a fairly neglected, but obviously exceedingly important area. Dr. Domino is an outstanding investigator and has a lot of experience with studying the effects of nicotine, and he is certainly an appropriate man to do the research proposed. This proposal is very wide ranging and the budget, while it may be large compared to others, is small for the amount of work he plans to do with a variety of species.

Unfortunately I did not get a vitae on Theodore Spaulding, so I do not know what his competence is. Since he is a key figure in this grant I think that should make a big difference.

The fact that nicotine will increase acetylcholine may be extremely important. The relationship between nicotine and acetylcholine has been a central question in pharmacology for a hundred years. I would certainly like to see Dr. Domino look into this matter.

Sincerely yours,


Murray E. Jarvik, M. D.
Professor of Psychiatry
and Pharmacology

mej rg

1003542000

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Walter B. Essman, M.D., Ph.D.

Principal Investigator, (summer 2/9ths)

100

\$ 7,662. *

Barbara Kornreich

100

\$12,700.

Fringe Benefits for B. Kornreich = 16%

2,032.

Technical

Technical Assistant (to be selected)

100

\$ 8,300.

Nancy Mulligan (Secretary)

100

7,900.

Fringe Benefits (16%)

2,592.

Sub-Total for A \$41,186.

B. Consumable supplies (by major categories)

Chemicals

\$ 950.

Radiochemicals

1,850.

Centrifuge tubes, rotors, supplies

2,300.

Gases

400.

Glassware

800.

Animals

750.

Sub-Total for B \$ 7,050.

C. Other expenses (itemize) (Travel)

7th Winter Conference for Brain Research,

Vail, Colorado, Jan. '74

International Congress of Physiological Sciences,

New Delhi, India, Oct. '74

American Physiological Society, New Jersey

Collegium Internationale, Neuropharmacology,

Paris, France, May '74

Local Travel Expense

Sub-Total for C \$ 1,838.Running Total of A + B + C \$50,074

D. Permanent equipment (itemize)

Automatic Dishwasher

\$ 750.

Beckman Centrifuge Roter

1,035.

Sub-Total for D \$ 1,785.E \$ 7,511.

E. Indirect costs (15% of A+B+C)

* This figure includes salary + fringe benefits. Total request \$59,370.

1003542026

In general, the goal of these proposed experiments is to relate central nervous system changes, defined at the synaptic level, which occur as a consequence of acute or chronic stress and/or the interaction of such responses with nicotine. There is a further goal involving the relationship of time course for regional synaptic changes occurring at the central level with specific peripheral systems as considered earlier in this discussion.

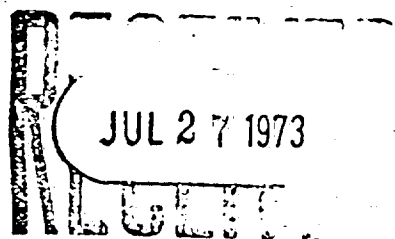
(9) Physical Facilities Available.

Two large air-conditioned laboratories and adjoining animal housing facilities are available and currently is use by the investigator; the laboratories are completely equipped with all equipment necessary for the biochemical procedures required, with the exception of those equipment items requested on the budget.

1003542044

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885



Application For Renewal of Research Grant
(Use extra pages as needed)

First Renewal ☒

Second Renewal ☐

Date: July 26, 1973

1. Principal Investigator (give title and degrees): Walter B. Essman, Ph. D., M.D.
Professor of Psychology & Biochemistry

2. Institution & address: Queens College of the City University of New York
65-30 Kissena Boulevard
Flushing, New York 11367
and
The Research Foundation of the City University of New York
1411 Broadway, New York, New York 10018

3. Department(s) where research will be done or collaboration provided:

Department of Psychology, Queens College of the City University of New York

4. Short title of study:

"Metabolic Response To Stress--Tobacco Smoke Interactions"

5. Proposed renewal date: January 1, 1974

6. How results to date have changed earlier specific research aims:

The initial aims set forth in this proposal have been essentially unchanged by any of the results obtained to date. It has become clear, however, that several additional sources of data have become available through the application of alternate methodologies to materials obtained from several of the experimental protocols outlined.

7. How results to date have changed earlier working hypothesis:

Results to date have not altered earlier working hypotheses; the data have, in fact, offered considerable support to the general theme of the project, indicating that at several levels of cellular regulation, an interaction can, in fact, be demonstrated between several varieties of behavioral and/or physiological stress and the effects of nicotine.

1003542024

CURRICULUM VITA

Edward Felix Domino

Personal

Born in Chicago, Illinois, November 20, 1924
Married: Antoinette Kaczorowski, November 20, 1948
Children: Karen Barbara, October 21, 1951
Laurence Edward, August 30, 1953
Debra Ann, November 20, 1956
Kenneth Edward, August 2, 1958
Steven Edward, October 24, 1961

Education

Primary: Chase Elementary School, Chicago, Illinois, 1930-1938
Secondary: Lane Technical High School, Chicago, Illinois, graduated 11th
in class of 990, 1938-1942
U.S. Navy Basic Radio Service Schools - Wright Junior College, Fall, 1943
Chicago, Illinois
Electronic Technician - Naval Research Laboratories, Anacostia,
D.C., 1943-1944
College and Professional Training:
B.S. - Division of Special Services
Two years credit in electrical engineering
Two years credit in premedicine, Univ. of Illinois, Urbana, 1948
B.S. - Bachelor of Science in Medicine, Univ. of Illinois, Chicago, 1949
M.S. - Pharmacology Univ. of Illinois, Chicago, 1951
M.D. - With honors Univ. of Illinois, Chicago, 1951
Internship - Rotating Presbyterian Hospital, Chicago, 1951-1952
Radioisotope Medical Qualification Course, Oak Ridge, Tennessee, July, 1968

Medical Licensures

Diplomate of the National Board of Medical Examiners, June 25, 1953, Cert.#26745
State of Illinois - March 11, 1953, Cert. #31818
State of Michigan - May 11, 1954, Cert. #20660
Schedule I, BNDD,# , University of Michigan
Schedule I, BNDD,# , Lafayette Clinic
Schedule II-V, BNDD #AD2734209, Private Physician
Schedule II-V, BNDD #PD0048911, University of Michigan Research
Schedule II-V, BNDD # , Lafayette Clinic

Permanent Positions and Experience

Electronic Technician, U.S.S. Pittsburgh, 1944-1946 (In charge of maintenance
and repair of search and fire-control radar)
Fellowships - During summer vacation months from medical school - 1949,
1950, Department of Pharmacology, Univ. of Illinois
Instructor in Pharmacology, Univ. of Illinois - 1951 to 1953, half time
basis; also half time on rotating internship

1003542015

Assistant Professor of Pharmacology, Univ. of Michigan - Sept. 1954 to Aug. 1958
 Associate Professor of Pharmacology, Univ. of Michigan - Sept. 1958 to Aug. 1962
 Professor of Pharmacology, Univ. of Michigan - Sept. 1962 to present

Visiting Professorships and Directorships

Visiting Associate Professor of Pharmacology, University of California,
 School of Medicine, San Francisco, California, October, 1961
 Visiting Director of Clinical Neurophysiology, Department of Neurosurgery,
 St. Barnabas Hospital, New York, September-October, 1963
 Visiting Professor of Pharmacology in Psychiatry
 Wayne State University, School of Medicine, October 1965 to present
 Director, Division of Neuropsychopharmacology and Michigan
 Neuropsychopharmacology Research Program at the Lafayette Clinic,
 Detroit, Michigan, June 1967 to present.
 Visiting Pharmacology, U.S.-U.S.S.R. Cultural Health Exchange, September 1971

U.S. Security Clearance

U.S. Navy - 1941-43
 Army Chemical Center - 1956-59

Administrative and Committee Functions, University of Michigan

Organization and responsibility for the major course in pharmacology taught
 to sophomore medical students by the Department of Pharmacology - 1960
 to 1967
 Member - Teaching and Curriculum Committee, Univ. of Michigan
 Graduate School, 1961 to 1967
 Member - Audio-Visual Committee, University of Michigan Medical School -
 1962 to 1967
 Organization and responsibility for the Senior Therapeutic Seminars -
 1964 to 1967
 Member - Committee Clinical Research Unit - 1965 to 1966
 Member - Coordination Committee on Senior Seminar - 1965 to 1967
 Member - Committee on Special Studies (Honors) Program - 1964 to 1968
 Director - Michigan Neuropsychopharmacology Training Program - 1966 to 1970
 Member - Committee for Integrated Teaching in Neural and Behavioral
 Sciences - 1966 to 1972
 Director - Michigan Neuropsychopharmacology Research Program - 1966 to present

Awards and Honors

Alpha Omega Alpha - 1951
 Sigma Xi Prize in Medicine - University of Illinois - 1951
 Title of Paper - Spinal Interneuron Depression by Benzazoles
 Research Award Michigan Society for Neurology and Psychiatry - 1955
 Title of Paper - Differential Drug Effects on the Brain Stem
 Activating and Diffuse Thalamic Projection Systems
 American Society of Anesthesiologists - First Prize, 1963
 Scientific Exhibit "Visually Evoked Response in Man: A New Technique
 for the Study of Drug Action" with Drs. Corssen and Sweet

1003542016

PUBLICATIONS

1. Fitzgerald, P.J., Li, T.G. and Yermakov, V. A comparison of the mass concentration of normal and carcinoma in situ cell of the human uterine cervix by means of the x-ray contact microradiography technique. X-RAY MICROSCOPY AND MICRORADIOGRAPHY. Ed. Cosslett and Engstrom, pp. 520-530, Academic Press, Inc., N.Y., 1957.
2. Fitzgerald, P.J., Yermakov, V., Sabin, L. and Levine, L. The mass of cancer in in situ cells of the human cervix uteri as compared to normal cervical cells. Acta Union Internationale Contre Le Cancer, Vol. XV-N. 2:296-302, 1959.
3. Yermakov, V. and Fitzgerald, P.J. Application of x-ray microradiography in Pathology. Bulletin of the New York Academy of Medicine, Vol. 36:778-482, 1960.
4. Lipkin, L.E., Yermakov, V. and Aronson, S.M. Ganglionic intracytoplasmic inclusion bodies: x-ray contact microradiographic and histochemical studies. AMA Arch. of Neur. 2:106, 1960.
5. Minkowitz, S., Soloway, H., Hall, Y.E. and Yermakov, V. Fatal hemorrhagic pancreatitis following chlorothiazide administration in pregnancy. Obst. Gyn. 24:337, 1964.
6. Bolooki, H., Margulies, M., Yermakov, V. and Gliedman, M. Portal hypertension with anomalies of inferior vena cava and hepatic vein. Arch. Surg. 94:267-270, 1967.
7. Coppola, A., Yermakov, V. and Caggiano, V. Pleomorphic lymphoma and gastric adenocarcinoma (collision neoplasm) associated with monoclonal macroglobulinemia and amyloidosis. A case report. Cancer, 23:576-585, 1969.
8. Perez, N., Rosen, Y., Lichtman, H. and Yermakov, V. Granulomatous gastritis of probably tuberculous etiology associated with megaloblastic anemia. Case Report. New York State Journal of Medicine. (In Press).

1003541978

that are coincident with the onset, duration, and adaptation to stress; (2) several of those changes which are utilized as indices of stress onset, permanence, or reversibility may be interacted upon by the effects of nicotine; (3) the nature of the biochemical or metabolic change observed within representative cellular model systems in response to either stress, nicotine, or their combination will be utilized as predictors of the onset of ensuing breakdown of functional metabolic systems; (4) the objective role of nicotine which serves to either alter the time course for onset of stress indices or changes the sequence over which they may occur or be modified will be assessed.

Details of Experimental Design and Procedures:

A well documented series of physiological changes resulting from acute stress, utilizing a considerable variety of stressor agents and/or events has been presented (Selye, 1950). On the basis of the work summarized and directed experience with one reliable stressor which easily meets the experimental needs for such a condition, we intend to utilize restraint-stress in rodents (mice, rats and guinea pigs) as the basis for the acute stress condition. A number of related variables concerning this form of acute stress have been defined in a number of studies completed in our laboratory, and these include such factors as food intake, time of day, age, etc., all of which can be controlled to provide conditions wherein this acute stress can be defined in terms of: (a) duration, and (b) frequency. The conditions constituting chronic stress have further been well documented in a series of experiments originating in our laboratory between 1964 and 1971 and these are basically involved with the utilization of isolation housing which constitutes the basis for chronic stress, the magnitude of which and adaptation to which can be titrated by a single variable of the duration of which the rodent is maintained under isolation. The proposed work rests upon the investigation of both peripheral as well as central cellular changes which accompany or follow stress and the resistivity by

1003542031

#917 - HUDGINS

1003542069

10. Essman, W.B.: Contributions of differential housing to brain development : some implications for sleep behavior. Maturation of Brain Mechanisms and Sleep Behavior. Washington, D.C.: U.S. Government Printing Office 1970d, (In Press).
11. Essman, W.B.: Some neurochemical correlates of altered memory consolidation. In: Trans. N.Y. Acad. Sci., 1970e, 32 : 948-973.
12. Essman, W.B., & Frison, J.D. Isolation-induced facilitation of gastric ulcerogenesis in mice. J. Psychosom. Res., 1966, 10, 183-188.
13. Essman, W.B., & Smith, G.E.: Behavioral and neurochemical differences between differentially housed mice. Amer. Zool., 1967, 7, 370.
14. Frisone, J.D., & Essman, W.B.: Stress-induced gastric lesions in mice. Psychol. Rep., 1965, 16, 941-946.

1003542050

Theodore A. Slotkin - Privileged Communication

b. Determination of the uptake and storage properties of the storage vesicles: Litters of rats will be sacrificed as described above, and adrenal homogenates will be centrifuged at 800 x g. Supernatants will then be used for determination of vesicular catecholamine fluxes. To determine the uptake capabilities of the vesicles, the suspensions will be incubated at 30° with either ^{14}C -epinephrine or ^3H -metaraminol, in the presence of ATP - Mg^{2+} as described previously (3, 4). The former amine is incorporated primarily by the reserpine-sensitive uptake mechanism, while the latter is incorporated primarily by the reserpine-insensitive mechanism (1, 2, 22, 23). The effluxes of endogenous and newly-incorporated amines from the storage vesicles will also be determined (1, 2, 3). Because "uptake" is a complex term (influx minus efflux), and since efflux is a measure of the stability of storage, only by the evaluation of efflux can an observed decrease in uptake be interpreted as a decrease in influx or a decrease in stability of storage (increase in efflux). These data should indicate whether there is a specific defect in uptake or storage of amines in hypertensive rats.

c. Buoyant density of storage vesicles: Catecholamines and nucleotides represent a significant fraction of the dry weight of the storage vesicles (24). Therefore, it would be expected that, if vesicles from hypertensive rats have altered CA or ATP levels, they might equilibrate at lower-than-normal densities on continuous sucrose gradients. The separation of lighter vesicles with lower CA contents in normal adult rabbits and rats after massive vesicle depletion has been described previously (4, 25); studies of this type will be carried out with vesicles from developing normotensive and hypertensive rats to see whether there are differences in buoyant density.

d. Depletion and repletion of adrenal amine stores: The ability of the adrenal glands of developing SHR and normal rats to respond to neural stimulation and to recover from massive stimulation will be tested by the administration of insulin (5 IU/kg); in normal adult rats, this results in depletion of adrenal CA to 20% of control levels within 4 hours (3), followed by a return to normal levels in 4 days (4). Should the ability to secrete amines be altered in the SHR, similar studies will be conducted in vitro using potassium as a secretagogue. If there is an in vitro response but only poor in vivo secretion after insulin, this could imply that the altered response to neural stimulation results from a presynaptic defect. If the gland responds poorly to both treatments, it would imply that any alteration in catecholamine secretion is due to a change in the ability of the adrenal to respond to neural input, either through interference with amine synthesis, storage, or secretion.

The rate of recovery of amines and vesicles after neural stimulation (insulin administration) or after non-neural depletion by reserpine (5 mg/kg) would give further information regarding whether the rate of CA and vesicle turnover is altered in hypertensive rats. For example, 50% of the vesicles lost during massive secretion are replaced within 24 hours in normotensive adult rats (4). It would therefore be worthwhile to study the rates of recovery in developing SHR and normotensive rats to determine if there is any alteration in the capacity to resynthesize vesicles which have been secreted.

Because of the likelihood of altered neural input in SHR, studies utilizing chlorisondamine (a ganglionic blocking agent) will be carried out: SHR and normals will be given twice daily injections (5 mg/kg s.c.) for one week and

1003541939

The neuropsychopharmacological effects of chronic nicotine in small doses differ from those on acute administration. The analogy is that of the first time exposure to tobacco smoking in man in contrast to its habitual use. It is proposed that many of the behavioral disrupting effects of nicotine observed on acute administration are either less obvious or more facilitating on chronic administration. Similarly, it is proposed that the effects of nicotine on brain acetylcholine and on EEG activation are altered more on acute than on chronic administration. Abrupt withdrawal of nicotine administration is predicted to have minor effects on these various behavioral and neuropharmacological endpoints in contrast to the well known actions of other psychoactive drugs.

9. Details of experimental design and procedures (append extra pages as necessary)

1. Behavioral studies in the rat.

Nicotine in doses equivalent to tobacco smoking in man will be given intraperitoneally to adult male albino Holtzman rats of approximately 90 days of age. The dose of nicotine is a critical variable and will be varied from 50 to 250 $\mu\text{g}/\text{kg}$. In initial experiments the intraperitoneal route will be used. If this route is not satisfactory in the chronic animals, the subcutaneous route will be used. There is considerable data in the literature (Domino, 1967; Morrison and Armitage, 1967; Driscoll and Bättig, 1970; Orsingher and Fulginiti, 1971; and Nelson and Goldstein, 1972) that this dose range (50-250 $\mu\text{g}/\text{kg}$) is commonly used in behavioral experiments in the rat and relevant to those taken by man during tobacco smoking.

The behavioral endpoints to be studied will include:

- a. Acquisition and performance of one-way shuttle box avoidance. Adult male albino Holtzman rats will be housed 2 to a cage with free access to food and water. They will be on a 12:00 p.m. to 7:00 a.m. dark and 7:00 a.m. to 12:00 p.m. light cycle. They will be trained in a one way electric shock avoidance procedure as described by Tenen (1966) and Caldwell *et al.* (1970). Trials will be presented on a variable interval schedule with a mean of 30 seconds. At the beginning of the CS, four red lights in the corners of the box will turn on and a movable wall which blocked the rats from jumping onto a ledge will move back. The rat will have 5 seconds to jump onto the exposed ledge. If the rat does not jump onto the ledge, a 1.0 ma electric shock will be applied to the grid floor (US) for another 5 seconds. Lights and shock overlap. At the end of the 5 seconds (no response) or when the rat jumps onto the platform the lights and shock will be terminated. After 30 seconds the wall will move to conceal the ledge and the animal will return to the grid floor to await the next trial. Both acquisition and performance will be tested. The naive animals will be divided into 6 per group. The nicotine in various logarithmic doses within the range of 50-250 $\mu\text{g}/\text{kg}$ will be given or comparable control vehicle and the animal given 50 trials. Mean acquisition will be expressed

1003542006

-25-

CURRICULUM VITAE

NAME: VALENTIN MICHAEL YERYAKOV

HOME ADDRESS
& PHONE NO.:

REDACTED

MARITAL
STATUS:

REDACTED

REDACTED

PLACE & DATE
OF BIRTH:OFFICE ADDRESS
& PHONE NO.:Institute of Pathology, Autopsy Service
Kings County Hospital Center, 451 Clarkson Ave.
Brooklyn 3, N.Y. IN 2 4000, Ext. 6658.PRELIMINARY
EDUCATION:First Russian "Archduke Constantine Constant-
inovich" Kadet Corps
Bela Crkva, Yugoslavia (Equivalent to Real
Gymnasium) 1932-1940.
Taconic Institute, 1949-50 (American Literature
and History).SUPPLEMENTAL
EDUCATION:

City College of New York - 1951 (Bacteriology)

PROFESSIONAL
EDUCATION:University of Belgrade, Yugoslavia - 1940-1941.
UNRRA University, Munich, Germany - 1945-1946
Rupert Carola University, Heidelberg, Germany -
1946-1949, 1952-1953.PROFESSIONAL
DEGREES:Physician - Rupert Carola University - 1953
Doctor of Medicine - Rupert Carola University -
1953.HOSPITAL
TRAINING:Rotating Internship, St. Clare's Hospital,
New York City, 1954-1955.
Resident, Pathology, Kings County Hospital
Center, Brooklyn, New York, 1955-1956.TEACHING
APPOINTMENTS:Damon Runyon Cancer Research Fellow at New York
State University, Downstate Medical Center -
1956-1957.1 Instructor in Pathology, College of Medicine,
Downstate Medical Center, S.U.N.Y. 1957-1962.2 Assistant Professor, College of Medicine,
Downstate Medical Center, S.U.N.Y. 1962-1963.3 Associate Professor, College of Medicine,
Downstate Medical Center, S.U.N.Y. 1963 -

1. Resident Pathologist, Kings County Hospital 1954-1955

2. Pathologist " " " " 1962-1970

3. Senior Resident Pathologist " " " " 1970 -

Hospital
Appointments:

1003541977

2.
8. Brief statement of working hypothesis.

It is well established that more smokers than non-smokers suffer from duodenal ulcers. Furthermore there is delayed healing of peptic ulcers in smokers as compared to non-smokers (3). The reason for this, however, remains unclear. In order to cause or maintain duodenal ulcers nicotine probably would have to increase duodenal acidity. This could be effected 1) by increased gastric secretion of HCl or 2) by decreased neutralization of a normal amount of gastric acid in the duodenum. The first possibility seems unlikely, since gastric acid secretion in smokers has not been found to be increased by most investigators (4-8). The latter possibility, however, has been supported recently by findings of Jacobson and his group (1,2). They reported that nicotine acutely inhibited pancreatic secretion of fluid and bicarbonate, the major buffers for acid in the duodenum. On the basis of these findings, it appears likely, that insufficient neutralization of acidic duodenal contents may be an important factor for the formation or maintenance of duodenal ulcers in smokers.

The mechanism by which nicotine inhibits pancreatic secretion is not known. Jacobson (9) speculated that at least part of the action of nicotine might be mediated by inhibition of the release of secretin, the major stimulus for pancreatic secretion of water and bicarbonate. Until recently this possibility could not be directly investigated due to the lack of adequate assay methods for the measurement of secretin in blood. We have recently developed a sensitive, specific and reproducible radioimmunoassay

9. Details of experimental design and procedures (append extra pages as necessary) (continued on next page)

I. Operative Procedures: The studies will be performed on overnight fasted healthy normal mongrel dogs weighing between 15 and 25 kg. The animals will be anesthetized by I.V. injection of Nembutal (approximately 6 mg/kg). They will be intubated and artificially respired. Laparotomy will be performed and polyethylene catheters will be inserted into both femoral veins and the portal vein, 1-2 inches distal from the portal area. Then, the minor pancreatic duct will be ligated and a small polyvinyl catheter will be inserted into the major pancreatic duct between the head of the pancreas and the duodenal wall. A double lumen tube will be inserted through a gastrotomy opening into the stomach, passed through the pylorus and its position will be stabilized 1-2 inches distal from the duodenal bulb.

II. Experimental Design:

A. Effect of nicotine or cigarette smoke on basal IRS secretion: Each group of experiments will include three test periods. After an initial control period (-15 minutes until 0 minutes) nicotine (12.5 - 50 mg/kg) will be infused for 30 minutes. This will be followed by another control period. In a separate group of experiments inhalation of cigarette smoke will replace the infusion of nicotine. To accomplish this, cigarettes will be taped to the end of the tracheal tube.

B. Effect of nicotine or cigarette smoke on HCl stimulated IRS secretion: Each experiment will include four test periods. After a brief control period (-15 minutes until 0 minutes) HCl (160 mmol in distilled water) will be infused at a rate of 4 ml per minute for 30 minutes. Nicotine (12.5 to 50 mg/kg) will be infused I.V. together with HCl. This will be followed by a rest period (30-60 minutes) and a second infusion of HCl (60-90 minutes). Again, in a separate group of experiments inhalation of cigarette smoke will replace the infusion of nicotine.

III. Laboratory Determinations:

A. Collection of blood. Preparation of serum: Blood samples will be obtained from portal and femoral veins at frequent intervals before, during and after nicotine and/or HCl infusion, allowed to clot and will be centrifuged at 4° C. Serum will be stored at -15° C until assayed.

B. Determination of immunoreactive secretin (IRS): IRS will be measured by a sensitive and specific radioimmunoassay which has recently been described in detail (10). (A reprint of this article is added).

C. Determination of pancreatic volume and bicarbonate: Volume of pancreatic fluid will be collected in 15 minute intervals. Bicarbonate concentrations will be measured by adding 0.5 ml of pancreatic fluid to 1.0 ml of 0.1 N HCl, bringing the mixture briefly to boil and back titrating the residual HCl with 0.1 N sodium hydroxide in an automatic titrator (pH 7.0). In addition, pH will be measured in samples of duodenal fluid collected at frequent intervals.

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Figure 1

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Item #9. Details of Experimental Design and Procedures

The effects of nicotine on systolic blood pressure, body weights, biochemical parameters, endocrine function and cardiovascular pathologies, etc. will be studied in test and control groups of 4 age levels (4 weeks, 6 months, 1 year and 1½ years). Body weights will be measured weekly and food consumption of aliquot groups will be recorded weekly. Blood pressure readings of the respective test and control experimental groups will be recorded after the first and 2nd weeks of nicotine administration and on alternate weeks thereafter.

At appropriate intervals prior to sacrifice urine collections will be obtained from the test and control spontaneously hypertensive and normotensive groups to evaluate urinary 17-ketosteroid (77) and urinary catecholamine (78) output. The following schema presents the format and population sizes of the various experimental studies:

Protocol: Four groups of SHR and NR to be sacrificed after 4 weeks, 6 months, 1 year and 1½ years of nicotine alkaloid administration (subcutaneously, twice daily in slow release preparations; dose 2.28 mg/kg/day.

Group I: 4 weeks	Test SHR-	30 rats
	Control SHR-	30 rats
	Test NR-	30 rats
	Control NR-	30 rats
	Total-	120 rats

Group II (6 months), Group III (1 year) and Group IV (1½ years) to consist of larger initial populations (35 per group) to compensate for experimental deaths. Total rats for the 4 groups-480 rats.

During the course of the respective experimental investigations at appropriate intervals, test and control SHR and NR will be sacrificed by rapid decapitation (Harvard decapitator) and blood samples will be collected in heparinized beakers for plasma corticosterone (79) glucose (80), total protein (81) and Na⁺ and K⁺ (82) assays. In addition, aliquot plasma samples will be analyzed for total cholesterol (83) and free cholesterol (83), plasma FFA (84), triglyceride (85) and phospholipid (86) titers. The adrenals will be rapidly excised, trimmed of fat and connective tissue and weighed prior to adrenal corticosterone (87) and adrenal catecholamine (88) analyses. Thus, the various biochemical tests associated with organ weights and histological data will furnish information concerning the responsiveness and differential effects of nicotine on the spontaneously hypertensive and normotensive rats. The assays will yield insight into adrenomedullary (catecholamine), adrenocortical (glucocorticoid and mineralocorticoid) and possibly gonadal (androgenic 17-ketosteroids) and the hormonal influences on glucose, fat and Na⁺ and K⁺ metabolic and regulatory processes, etc. Histological preparations of the pancreas and pituitary will yield further information concerning their respective hormonal products. During the various autopsy periods care will be exercised to check for gross pathologies and to approximate amounts of intradermal fat in the respective test and control groups. Such organs as the adrenals, heart, liver, spleen, thymus, kidneys, testes, seminal vesicles, lungs and brain will be removed for organ weight analyses, in addition to being checked for gross pathology. All organs will be weighed on a Sartorius Selecta

1003541964

THEODORE C. SPAULDING

SCIENTIFIC PAPERS

1. Spaulding, T.C., Ford, R., Dewey, W.L., McMillan, D.E., and Harris, L.S. Some pharmacological effects of phenitrone and its interactions with Δ^9 -THC. Europ. J. Pharmacol. In press.
2. Cocolas, G.H., Robinson, E.C., Dewey, W.L., and Spaulding, T.C., The preparation and activity of some beta-substituted acetylcholine iodides. J. Pharmaceutical Sci. 60, 1749-1752 (1971).

ABSTRACTS

1. Cocolas, G.H., Robinson, E.C., Dewey, W.L., and Spaulding, T.C. Preparation and activity of α - substituted acetylcholine iodides. Sixth Annual Southeastern Medicinal Chemistry Meeting in Miniature. School of Pharmacy, University of South Carolina, March-10 - 11, 1972.
2. Spaulding, T.C., Dewey, W.L., Harris, L.S. The pharmacological effects of and the lack of Δ^9 -THC blocking activity of phenitrone. Pharmacologist 13, 296, 1971.
3. Cocolas, G.H., Robinson, E.C., Dewey, W.L. and Spaulding, T.C. Molecular complementariness at the muscarinic receptor. Fifth Annual Southeastern Medicinal Chemistry Meeting in Miniature. School of Pharmacy, University of North Carolina, March 19 and 20, 1971.
4. Cocolas, G.H., Robinson, E.C., Dewey, W.L., and Spaulding, T.C. Molecular complimentariness at the muscarinic receptor. 23rd International Congress of Pure and Applied Chemistry. pg. 76 (1971).

1003542021

titrated against the duration and frequency of nicotine treatment. Furthermore, the sequence, as well as the interval between stress and nicotine treatment will be varied; i.e., stress either proceeding or following treatment at intervals ranging from 10 minutes to 24 hours, or if the emerging data so indicates, the two variables will be superimposed in time. Nicotine, as the alkaloid will be used in acute or chronic doses ranging from 0.1 - 0.8 mg/kg., i.p. As indicated above, for those studies concerned with one of the four peripheral systems to be investigated (cardiac tissue), the other three peripheral sources will be simultaneously investigated utilizing the same animals from which the cardiac tissue is obtained; i.e., adrenal tissue, platelets, and gastrointestinal tissue.

(2) Adrenal Tissue.

The relationships between adrenal morphology and chemistry and the concept of "emotionality", as well as consequences of stress, have been known for some time. Rats selectively bred for high levels of "emotionality" have been shown to have heavier adrenals (Yeakel & Rhoades, 1941) and among wild rats showing both greater "emotionality" levels as well as an increased degree of reactivity to environmental cues, heavier adrenal weights were also demonstrated (Rogers & Richter, 1948). The "reactivity" of adrenal tissue to stress has been clearly documented (Selye, 1950) and the nature of the storage and release mechanisms upon which the active constituents of this tissue depends has also been extensively studied (Blaschko, 1954). The nature of the catecholamine storage granules in adrenal tissue has presented an extremely interesting conceptual framework within which mechanisms operative both during and/or following stress may be further studied. The chromaffin granule and its catecholamine-binding protein, chromogranin, have offered considerable promise as mechanisms which provide further insight into those processes which affect the synthesis, storage, and release of adrenal catecholamines. The techniques for isolation of both the chromaffin granules, which are totally adrenergic in content, have been accurately worked out utilizing differential and density gradient

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centrifugation methods (Blaschko, et. al., 1955). It will be the object of the proposed investigation, in dealing with isolated chromaffin granules from rodent medulla, during and following both acute and chronic stress, to assess changes in catecholamine content and turnover. It has been shown that the effect of smoking upon the release of epinephrine appear dependent upon the duration of inhalation or dosage, as well as upon the interval and probably frequency thereof, intervening between successive treatments (Watts, 1960). The premise upon which observed changes in urinary excretion of catecholamines in man during smoking is based is that this reflects adrenal medullary secretion. There appears to be no systemic evidence relating the effects of nicotine, to specific changes in the storage granules in the adrenal tissue. It will be the purpose of this series of experiments to describe the interaction of stress and nicotine, investigated within the context of the previously outlined experimental paradigm, with a view toward defining this interaction on the basis of changes in catecholamine synthesis, storage, and release mechanisms which operate during these events in the chromaffin granules of the adrenal.

(3) Platelets.

The known and suspected role of platelets in the maintenance of homeostasis are well documented (Kowalski & Niewiarowski, 1967; Johnson, et. al., 1961; Mustard & Packham, 1970). Furthermore, the pathological states leading to infarcts involving platelet aggregation and adhesion are known (Mustard & Packham, 1970). Various stressor events presented to rodents, such as physical restraint or social deprivation (acute and chronic stress) produce a decrease in circulating levels of monodisperse platelets, presumably as a result of aggregation. In humans, various investigators have shown that cigarette smoking increases platelet adhesiveness (Mustard & Murphy, 1968; Mustard & Murphy, 1963; Schievelbein & Werle, 1962).

restraint stress in cardiac necrosis (Selye, 1950), and typical stress cardiopathy, consisting of disseminated micronecroses has been more extensively studied (Selye, 1961) and likened to cardiopathy as developed with isoproterenol and catecholamines. The biochemical commitants of such stress-induced cardiac pathology have not been documented nor has the change in the time course of onset as potentially indicated for nicotine treatment been studied within this context. It seems apparent, in view of the foregoing evidence, that acute and possibly chronic stress, and their potential interaction with nicotine would seem extremely likely as conditions for the modification of norepinephrine storage pools measured in cardiac tissue. Use, therefore, of such storage pool ratios will be made in assessing the onset, duration, recovery, and/or adaptation to acute stress (restraint) or chronic stress (isolation).

In as much as the vagal innervation of the heart is mediated by cholinergic endings in that tissue, an avenue for consideration of synapses maintained by acetylcholine is provided for study. Isolation of such cardiac synaptosomes by differential and density gradient ultracentrifugation will provide a basis for study of the synthesis, uptake, storage, release, and turnover occurring at cardiac cholinergic endings. The effects of stress (acute or chronic) upon cardiac cholinergic innervation and metabolism have not been studied, as have not the effects of nicotine upon this system; although, in the latter instance, the cholinergic effects of nicotine would warrant consideration of this potential source of interaction with stress at cholinergic endings in the heart.

The conditions under which stress-nicotine parameters will be regulated will consist of treating both conditions as independent variables within the context of a pharmacological paradigm, i.e., the duration of acute or chronic stress will be

1003542036

1. Research on Anticarcinomatous Substances. I. A number of 1-bromo 7-methoxyacridine and 3-bromo-7-methoxyacridine derivatives. Biological Part.-Polish Pathology (Patologia Polska) 1958, 9, 4, 331-343.
2. Properties of the fibrinous membrane produced in Poland.-The Polish Physician Journal (Polski Tygodnik Lekarski) I 59, 14, 26, I-8.
3. Influence of the somatotropic hormone (STH) on the body weight and tumor growth of mice with transplantable Crocker sarcoma.-The Polish - Physician Journal (Polski Tygodnik Lekarski) 1960, 15, 9, 2-7.
4. The Action of some Acridine Derivatives on the growth of Crocker sarcoma in mice. Polish Medical Science and History October, 1960, 3, 4, 154-166.
5. Isonicotinic acid Hydrazide (INH) as a carcinogenic agent in mice. First Report. Polish Pathology (Patologia Polska) 1961, 12, 1, 53-56.
6. Isonicotinic acid hydrazide (INH) as a carcinogenic agent in mice. Second Report. Polish Pathology (Patologia Polska) 1962, 13, 2, 185-194.
7. Influence of hormones of the suprarenal cortex on the transplantable Crocker sarcoma. Societas Scientiarum Gedanensis. Acta Biologica et Medica. 1962, II, 341-363.
8. Haemanagiomatosis diffusa hepatis with thrombocytopoenia. - The Archiv of Polish Internal Medicine (Polskie Archiwum Medycyny Wewnetrznej) 1964, 34, 6, 781-784.
9. Heart failure due to leukemic infiltration of the heart in a patient with myelosis leukemia.-The Archiv of Polish Internal Medicine (Polskie Archiwum Medycyny Wewnetrznej) 1964, 34, II, 1399-1492.
10. Two cases of giant hypertrophy of the gastric mucosal rugae (Menetriers Disease). Polish Review -f Radiology and Nuclear Medicine (Polskie Archiwum Radiologii i Medycyny Nuklearnej) 1965, 29, 2, 149-156.
11. Rheumatic pneumonia in a six year old child.-Polish Pathology (Patologia Polska) 1965, 16, 3, 367-371.
12. Isonicotinic acid hydrazide (INH) as a cancerogenic factor. III Report.- Polish Pathology (Patologia Polska) 1967, 28, 2, 295-300.
13. The comparing of the clinical and histopathological picture in Hirschsprungs Disease. - Memorial of the 44 Polish Surgeons Congress in Krakow (Pamiętnik 44 zjazdu Chirurgów Polskich w Krakowie) 26-28.9.1968, 440-441.

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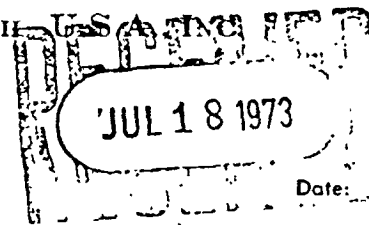
PHARMACOLOGY

Comm.

Dr. Bing
Dr. Gardner
Dr. Jacobson

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A. INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885



Application for Research Grant
(Use extra pages as needed)

Date: 7/11/73

1. Principal Investigator (give title and degrees):

Guenther Boden, M.D.

Assistant Professor of Medicine and Assistant Director of the General Clinical Research Center

2. Institution & address:

Temple University Health Sciences Center
3401 N. Broad Street
Philadelphia, Pennsylvania 19140

3. Department(s) where research will be done or collaboration provided:

Department of Medicine, Temple University Health Sciences Center

4. Short title of study:

Effect of Nicotine and Cigarette Smoke on Secretin Secretion

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: 2 years

7. Brief description of specific research aims:

Specific Aims: Previously published data suggest that nicotine inhibits pancreatic secretion of water and bicarbonate (1,2). The specific aims of the proposed study are: 1) to determine whether nicotine or cigarette smoke do indeed inhibit pancreatic secretion of water and bicarbonate and 2) if so, to determine whether the inhibition is mediated by the gastrointestinal hormone secretin or by a direct effect on the pancreas or both. To do this we intend to study the acute effects of intravenously infused nicotine or inhaled cigarette smoke on basal as well as HCl stimulated serum secretin concentrations and on pancreatic volume and bicarbonate secretion in dogs.

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has not been studied systematically and certainly it would appear particularly relevant to consider whether the presence of "free" cardiac norepinephrine can be utilized as an index of stress, inasmuch as changes in the total content of this catecholamine certainly have indicated (Selye, 1950). Therefore, it would seem quite reasonable to anticipate that conditions imposed upon the mechanisms of synthesis, uptake, binding, or catabolism could easily affect the cardiac amine storage pools. With the availability of techniques for subcellular fractionation of cardiac tissue (Michaelson, et. al., 1964; von Euler & Lishajko, 1965) and separation, thereby, of storage pools from homogenized cardiac tissue, determinations will be made of "free", to "bound" ratio of this cardiac catecholamine in order to assess the quantitative relationship between acute and chronic stress and the interaction therewith of nicotine treatment and changes in the rates of these storage pools. There are some bases for a consideration of cardiopathies which reside within observations, on one hand of cardiac catecholamine depletion in heart failure induced experimentally, or observed clinically (Chidsey, et. al., 1964; Chidsey, et. al., 1963; Chidsey, et. al., 1966) and increases in the cardiac liberation of norepinephrine associated with myocardial infarction (Kuschke & Schneider, 1960) and in cardiac decompensation (Kuschke, 1961). The production of focal myocarditis and hemorrhagic lesions localized to the pericardium and endocardium have been observed following injection of norepinephrine in dogs (Szakacs & Cannon, 1958), as well as in post mortum examination following therapeutic infusion of norepinephrine. An increase of liberation of norepinephrine from cardiac tissue has, as previously indicated, been well established in both experimental animal studies (Selye, 1950) as well as in man (Elmadjian, et. al., 1956; Elmadjian, et. al., 1957) and in addition to this, the interesting observation that the release of cardiac norepinephrine may also be accomplished by nicotine (Westfall & Watts, 1964). One pathophysiological consequence which has been demonstrated in cardiac tissue as a consequence of

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8. Brief statement of working hypothesis:

2.

Chronic nicotine treatment produces shifts in the balance between reticular formational and limbic influences on arousal, resulting in a state of enhanced "motivational arousal" and reduced "drive arousal". Such changes in brain functional mechanisms should produce the qualitative state of arousal appropriate for engaging in goal-directed behavior. Further, the shift in the balance between the subcortical system influencing arousal (in the "chronic nicotine state") should modify both the behavioral and electrophysiological effects of psycho-active drugs.

9. Details of experimental design and procedures (append extra pages as necessary)

See attached pages.

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#929 - LEETE

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is the synapse; the response of the synaptic region and the functions which these units serve will be investigated under both conditions of acute and chronic stress. Such consideration will involve a relationship of synaptic events to observe peripheral changes, considered on the basis of their onset, duration, and recovery with a view toward specifying some of the neurobiological consequences of the interaction of stress with nicotine treatment.

With the advantage of having available techniques for the subcellular isolation and characterization of pre-synaptic nerve endings (synaptosomes) from various regions of the mammalian brain (Whittaker, 1970) the feasibility of utilizing the synaptosome as a model for the central consequence of the proposed interaction appears highly warranted. Specifically, those synaptic events to be considered, in terms of characterization of their unique capacity to utilize a given putative transmitter molecule, will be those cholinergic and adrenergic units that are temporally and functionally linked to the stress response. It is felt that statements concerning the regional, cellular, and subcellular events which participate in synaptic transmission in terms of their contribution to the synthesis, uptake, storage, and release of molecules possessing unique neurobiological properties, may be made for stress effects, nicotine effects, as well as their interaction.

The parameters involved with the experimental procedures will be those outlined earlier in the design and several areas of the rodent brain will be specifically considered in view of their morphological and functional interrelationship to one another. Specifically, the cerebral cortex, limbic system, and cerebral cortex will be utilized for the fractionation procedures in which synaptosomes will be prepared; further fractionation of these units will be done in order to derive cytoplasm, membrane, vesicular, and mitochondrial fractions. The details of such fractionation as well as the rationale underlying structural specificity and biochemical individuality of these systems is supported in some of the appended material.

7.
7.(b)

at sustained, reproduceable levels on a difficult visual attention task. We found that chronic nicotine treatment did improve the efficiency of responses to goal-oriented stimuli above the control optimal levels without causing or being accompanied by a general, non-specific increase in behavioral activity (Nelsen and Goldstein, *Psychopharmacologia* 26:347-360, 1972). The results of these studies invited further research directed both towards a better understanding of the mechanisms and nature of arousal and of the motivations accounting for the widespread self-administration of nicotine by humans.

If as Routtenberg has suggested (and our results imply), there exists a balance between the limbic and RF influences on arousal, it would follow that modification of the relationships toward greater limbic system control (as in the "chronic nicotine state") should alter the sensitivity of the RF arousal pathway to manipulation. Our specific aims are to test this hypothesis directly using both electrophysiological and pharmacological tools. It is well demonstrated that electrical stimulation delivered to the RF produces cortical activation and that the current necessary to elicit the response varies depending on the state of the brain. We propose to characterize the changes in sensitivity of reticular-cortical relationships during the "chronic nicotine state" via studies of the threshold for and duration of cortical activation in chronically nicotine-treated and saline-treated rats. A number of stimulant and/or psycho-active drugs are known or

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Presumably, the active constituent in cigarette smoke is nicotine (Werle & proposed investigation, in dealing with isolated cigarette smoke, Werle & Schievebein, 1965). Little is known of the interaction of stress and nicotine on platelet physiology or biochemistry. Those tissue constituents, serotonin, norepinephrine, epinephrine and histamine, selected for investigation in this study are all capable of increasing platelet aggregation alone, or potentiating the action of one of the others (Mustard & Packham, 1970). However, in the case of serotonin, it has been shown that a tachyphalaxis develops; this furthermore produced tachyphalaxis to the aggregating action of epinephrine and ADP (Baumgartner & Born, 1968). These aggregating-inducing agents, which can act in vivo as well as in vitro, promote the release of certain platelet constituents which act to cause platelet lysis (Davey & Luscher, 1968; Holmsen, et. al., 1969). The constituents of interest to this study are histamine, catecholamines, serotonin and lysosomal enzymes.

The localization of the former agents in platelets is thought to represent a means by which the circulating levels of these vasoactive agents are kept low, until the enzymes for synthesis are not present (Shore, 1962). The paradox of this situation is that those agents, epinephrine, serotonin and histamine are released from other sites during stress and in an attempt to reduce circulating levels, platelet uptake occurs. As has been shown for serotonin, it is the uptake process which causes aggregation and release of platelet constituents. The uptake requires the utilization of ATP which produces ADP, which in turn is the causative factor in platelet aggregation. Furthermore, the uptake process is not particularly specific for serotonin, but also operable for other amines and provides thereby the basis for the false transmitter concept (Kopin, 1966). These observations provide the basis for the hypothesis that platelet serotonin (the major amine present) is released as part of the initial stress reaction. The released serotonin

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Brief Description of Objectives or Specific Aims:

With the development of several methods in cellular biology, molecular biology, and neurobiology, it has become increasingly more possible to evolve cellular and subcellular model systems within which aspects of such processes as development, aging, and pathology may be studied; as a direct consequence of being able to utilize simplified model systems of this type considerable economy of technology, theory, and generation of testable hypotheses has been achieved. One purpose in the organization of this general research program is to investigate, within the context of such model systems, the effects of acute and chronic stress; the basis for the selection of the model systems within which stress effects would be investigated resides within, generally, a distinction between peripheral and central nervous system models and measures, and these will be utilized as a descriptive and predictive means of assessing acute and chronic stress effects, in terms of onset, duration, and adaptation. Within this same context it will be the purpose of the proposed investigation to view the interaction of acute and chronic stress effects upon cellular model systems and the acute and chronic treatment with nicotine, the latter being considered within the context of a pharmacological event.

The proposed project is directed toward the evaluation of the nature and sequence of metabolic and biochemical changes resulting from stress and/or its systems showing adaptation to stress and their interaction with the effects produced by nicotine. The basis of the objectives of this proposed work is a definition of those cellular and subcellular units, the biochemical or metabolic composition of which, may be altered by either stress, nicotine treatment, or the interaction of the two. The indices of stress and the interactive effects thereupon contributed by nicotine treatment will reside within a consideration of biologically active molecules which characterize a cellular or organelle system with which they are classically identified. As such, changes in the content, storage pool levels, or

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9. (e) design and the results will be tested by applying an analysis of variance.

The attached progress report includes the description of the study designed to test the electroencephalographic effects of acute stimulant or psycho-active drug "challenges" in rats treated chronically with either nicotine or saline. This study has been conducted as per the design presented and is presently under analysis.

Kornetsky, Conan and Eliasson, Mona: Reticular Stimulation and Chlorpromazine: An Animal Model for Schizophrenic Over-arousal. Science 165:1273-1274, 1969.

Nelsen, Judith M.: Single Dose Tolerance to Morphine Sulfate: Electroencephalographic Correlates in Central Motivational Systems. Unpublished Doctoral Dissertation. (Boston University) 1970.

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20. Murphy, E.A., & Mustard, J.F. (1960). Nat. Cancer Inst. Monograph, 28: 47-56.
21. Mustard, J.F., & Packham, M.A. (1970). Pharmac. Rev. 22: 97-188.
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24. Rogers, P.V., & Richter, C.P. (1948). Endocrinology, 42: 46.
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28. Szakacs, J.E., & Cannon, A. (1958) Amer. J. Clin. Pathol., 30: 425.
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1003542046

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

July 26, 1973

Grant application No. 642C

To: The committee comprising Drs. Bing, Jacobson and Meier

Subject: Leonide Goldstein, D.Sc., Institute for Mental Health Sciences,
CMDNJ, Rutgers
Continuation Application No. 642C (no commitment)
"Behavioral and Electrophysiological Effects of the Chronic
Nicotine State in Rats".

History

This applicant has been supported by CTR since 1968, initially at the New Jersey Neuropsychiatric Institute, since November, 1972 at Rutgers. Dr. Judith M. Nelsen joined in 1970, as a recent Ph.D., to strengthen EEG techniques.

The current level of support is approximately \$29,000. a year.

Application 642C requests \$33,350. We have no commitment. As no additional years are projected, this apparently is a terminal request.

Documents Submitted (attached)

1. Application dated July 16, 73.
2. Progress Report No. 1 (EEG studies January 1, 1973 through June 1, 1973; behavioral pilot study, May 15, 1973 through July 1, 1973).

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Drs. Bing
Jacobson
Meier

PHARMACOLOGY

#6420

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

JUL 23 1973

Date: July 16, 73

1. Principal Investigator (give title and degrees): Leonide Goldstein, D. Sc. Associate Professor of Psychiatry, College of Medicine & Dentistry of New Jersey
Judith M. Nelsen, Ph. D. Instructor in Psychiatry. College of Medicine & Dentistry of New Jersey.

2. Institution & address: Institute for Mental Health Sciences CMDNJ Rutgers Medical School. P.O. Box 101 Piscataway, NJ 08854

3. Department(s) where research will be done or collaboration provided: Department of Psychiatry

4. Short title of study: Behavioral and Electrophysiological Effects of the "Chronic Nicotine State" in Rats.

5. Proposed starting date: January 1, 1974 (Actually, proposed renewal starting date for Grant # 642 B).

6. Estimated time to complete: One year

7. Brief description of specific research aims: See attached pages.

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which changes in interaction with nicotine occur. Specifically in peripheral tissue, those models of direct functional significance to be studied are as follows: (1) cardiac tissue (norepinephrine, acetylcholine); (2) adrenal tissue (epinephrine); (3) platelets (5-hydroxytryptamine); and (4) gastrointestinal tissue (5-hydroxytryptamine).

(1) There are a number of biologically active molecules which, through several mechanisms of action, serve to provide for either the elevation or reduction of selected metabolic substrates indicated above as specific for given tissue sites. These drugs will be utilized in experiments wherein a given biologically active molecule is either increased or decreased for physiologically availability at this site. Thereby, a tissue-specific, molecule-specific state may be imposed, by which the effects of stress and/or nicotine treatment may be assessed. It is specifically the purpose of this methodological approach to provide for increases or decreases in trophic and/or transmitter mediation of substrates specific to given storage granules in those tissues outlined above. Those substrates proposed for this use consist of (1) metaraminolbitartrate which displaced norepinephrine, inhibits its uptake by norepinephrine containing storage granules, and thereby increases norepinephrine availability and release. This drug would thereby appear appropriate to provide for increased norepinephrine availability and release in cardiac tissue. A dose by which this effect may be achieved is 15 mg/kg.

(2) α -methyl- p -tyrosine is a substance with which leads to the inhibition of tyrosine hydroxylase the rate limiting enzyme in the biological synthesis of dopamine and norepinephrine. A dose of 80-100 mg/kg of this compound will lead to tissue depletion of these catecholamines of about 60% within five hours and maintain reduced tissue levels for approximately 90 minutes. Such treatment would again be effective in reducing endogenous levels of cardiac tissue catecholamines, which would

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12. Boden, G., Lundy, L.E. and Owen, O.E.: Influence of levodopa on serum levels of anterior pituitary hormones in man. Neuroendocrinology 10:309-315, 1972.

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9. (a) The details of experimental design and procedures are essentially the same as those presented in our previous grant application (dated October 20, 1972) since that proposal was designed to include two years of research activity commencing January 1, 1973. The following reviews these experimental plans and includes the additions and changes we have incorporated. Two related lines of investigation are planned. The first concerns electroencephalographic measures of cortical activation induced by electrical stimulation via the mesencephalic reticular formation (RF) arousal pathway and by pharmacological agents. In previous applications for "Research Grants" from the Council (dated November 25, 1970 and October 25, 1971), we have reviewed the surgical and EEG measurement technics which we have developed for application to such studies. We propose to prepare with cortical and subcortical (RF) electrodes an experimental group of approximately 20 rats (Holtzman, Sprague-Dawley, male). These animals will undergo adaptation training in an EEG recording chamber. They will then be assigned in random fashion to two groups, one of which will receive chronic treatment with nicotine (100ug/kg, s.c., t.i.d.) and the other, with physiological saline.

A schedule for delivery of small doses of electrical stimulation to the RF will be carried out to determine the threshold for and the duration of cortical activation resulting from direct stimulation of the RF arousal pathway under conditions of chronic nicotine and chronic saline treatment. Arousal (activation) will be measured and quantified from the cortical EEG recordings. Further, comparisons between the

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7. (c) suspected to exert their CNS effects primarily by their actions at the level of the RF. Consequently, the alteration of cortical-limbic-reticular relationships resulting from chronic nicotine treatment should in turn modify the efficacy of these drugs. We have already conducted on study aimed at elucidating these effects by use of electroencephalographic measures. (Please see the attached "Progress Report" for details.)

A further aim is to extend studies beyond the measurement of electroencephalographic effects at cortical and subcortical levels to include the detection of the behavioral or functional consequences of the electrophysiological and pharmacological manipulations. Because it have been demonstrated to be sensitive both to the level and qualitative nature of arousal, we propose to use the visual attention task described previously and reviewed in "item 9" to quantify the behavioral consequences of alterations in brain structure relationships.

1003542060

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

July 31, 1973

Grant application #836AR1
PHARMACOLOGY

To: The committee comprising Drs. Bing, Jacobson and Meier

Subject: Walter B. Essman, Ph.D., M.D., Queens College, N.Y.
Renewal application 836AR1
"Metabolic Response to Stress -- Tobacco Smoke Interactions"

History

This study has been supported by CTR since 1971 (Essman's ongoing study of memory consolidation has been supported since 1968).

Last year, for this stress study, \$53,407. was requested; \$40,000. was awarded. Essman was notified of two additional years "priority in competition", at amounts not to exceed \$40,000. per year.

Application #836AR1 requests \$59,370. This exceeds not only the \$40,000. per year stipulated by CTR but also the \$56,395. originally estimated for this year.

The current grant ends September 30, 1973. If an award is recommended, it will be necessary to provide interim funds to adjust to the January 1, 1974 starting date.

Documents Submitted

1. Attached is application dated July 26, 1973 (26 pages).
2. Also attached is Progress Report #1, October 1, 1972 to June 30, 1973.
3. Reprints or manuscripts of the publications listed in item #11 of the application have been provided.

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Attachment


F.W.N.

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#868R1 - MCKENNIS

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(d) have defined as: 1) for o.e.'s, #o.e./# reinforcements times $100 \leq 30\%$, and 2) for c.e.'s, #c.e./# reinforcements times $100 \leq 30\%$. After the rats have reached criterion levels of performance, half the group will be subjected to chronic treatment with nicotine (100ug/kg, s.c., t.i.d.) and half to physiological saline. After approximately one week of regular injections, a schedule of low current level electrical stimulation to the RF will be introduced during the testing sessions. Such stimulation has been shown to disrupt performance in a similar task (Kornetsky and Eliasson, 1969) where electric foot-shock was the reinforcing agent. Since based on our previous work, differential baseline behavior is expected depending on whether rats are nicotine- or saline-treated, each group (in fact, each animal) will act as its own control. Our hypothesis is that because nicotine-treated rats are in a state of greater incentive-oriented arousal (hippocampal predominance in the control of cortical function), their performance will be affected less detrimentally by RF stimulation than the performance of saline-treated animals.

Further, behavioral studies are planned which will focus on the possible protective action of the chronic nicotine state on the disruptive effects of pharmacological agents known or suspected to act on the RF arousal pathway by causing a relative increase in general or drive-oriented arousal and hence, decrease in incentive-oriented arousal. These agents will include D-amphetamine, L.S.D., physostigmine, and tetrahydrocannabinol. They will be administered according to a randominized block

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It is hypothesized that if nicotine stimulates nicotinic cholinergic receptors in the brain the utilization of brain acetylcholine will be decreased.

In future phases of this grant isotopic labelling of choline as a direct measure of brain acetylcholine will be used, depending upon the results obtained.

5. Effects of chronic nicotine administration on neocortical and limbic system activation in the cat.

Bhattacharya and Goldstein (1970) have reported that in rabbits the subcutaneous administration of 200 $\mu\text{g}/\text{kg}$ of nicotine for three weeks caused a shift of EEG activation from the reticular formation to the hippocampus. This important finding needs to be replicated in other species of animals including the cat and monkey. Experiments will be performed initially using adult cats of both sexes with chronic indwelling brain electrodes. Surgical preparation of the animals will be under pentobarbital anesthesia. Stainless steel wires of 0.22 mm diameter, insulated except for the tips, will be used as the depth electrodes. Bipolar depth electrodes will be inserted into the amygdala, hippocampus, and reticular formation. Neocortical electrodes will be placed epidurally in the somatosensory and visual cortex. Each electrode will be soldered to a Cannon plug and fixed to the calvarium by dental cement. Silastic tubing, 0.7 mm diameter, will be inserted into the jugular vein and the other end fixed on top of the calvarium. The animals will be given a 2 week period to recover and given antibiotics prophylactically. Nicotine in doses of 10 $\mu\text{g}/\text{kg}$ i.v. will be given 4 times daily for a 2 week period and the EEG changes monitored before (to 0.9% NaCl injections), during, and after nicotine administration. Similar experiments will subsequently be performed in the monkey.

References

Bhattacharya, I.C. and Goldstein, L.: Influence of acute and chronic nicotine administration on intra- and inter-structural relationships of the electrical activity in the rabbit brain. *Neuropharmacology* 9: 109-118, 1970.

Caldwell, D.F., Oberleas, D., Clancy, J.J., and Praasad, A.S.: Behavioral impairment in adult rats following acute zinc deficiency. *Proc. Soc. Exp. Biol. Med.* 133: 1417-1421, 1970.

Domino, E.F.: Electroencephalographic and behavioral arousal effects of small doses of nicotine: A neuropsychopharmacological study. *Ann. N.Y. Acad. Sci.* 142: 216-244, 1967.

Domino, E.F. and Wilson, A.: Psychotropic drug influences on acetylcholine utilization. *Psychopharmacologia* 25: 291-298, 1972.

Driscoll, P. and Bättig, K.: The effect of nicotine and total alkaloids extracted from cigarette smoke on avoidance behavior in rats under extinction procedure. *Psychopharmacologia (Berl)* 18: 305-318, 1970.

1003542009

Item #7. Brief Description of Specific Research Aims

There have been conflicting reports regarding the association of smoking with blood cholesterol levels. Several investigators (7,35) have reported higher values in smokers, but Blackburn et al. (1) did not observe a statistically significant difference. Others (36, 37) have reported a statistical relationship between cigarette smoking and elevated serum lipids. Kershbaum et al. (38, 39) has demonstrated that free fatty acids are rapidly mobilized in man and dogs after cigarette smoking or nicotine administration. These changes resulted from the nicotine stimulated secretion and release of adrenal catecholamines (40). The possibilities of heightened levels of blood cholesterol, lipids and free fatty acids due to smoking or nicotine have significance in view of claims of direct relationships between smoking and atherosclerosis. Moreover, it has been reported that rabbits fed a cholesterol diet and administered nicotine showed an increase in serum cholesterol and the degree of aortic atherosclerotic lesions (41). It should be noted that Kershbaum et al. (8) reported significant increases in the serum cholesterol levels of dogs administered nicotine for 6 weeks but no significant changes in triglyceride levels. However, Wenzel and Beckloff (42) reported that rabbits administered nicotine and fed a minimal (0.1%) cholesterol diet showed significant increases in both plasma cholesterol and phospholipid.

Other biochemical investigations have similarly been diverse. Whereas, Blackburn et al. (1) reported higher fasting blood sugar levels in smokers, Roth and Schick (3) claimed that fasting blood sugar levels did not rise appreciably after and during smoking. Milton (43) has reported that low doses of nicotine considered to be in the smoking range increased blood sugar and mobilized non-esterified fatty acids in cats due to increased catecholamine secretions. The possible involvement of other hormonal systems must however be considered in relation to glucose metabolism and the carbohydrate metabolic processes. Thus plasma glucocorticoid output (acute study) which also controls carbohydrate metabolism was stimulated probably as a secondary effect of catecholamine release. Recent reports (44) have also cited the "high" concentrations of nicotine inhibit glucose-induced insulin secretion, while "lower" doses stimulate insulin secretion. Our histological study of the pancreas should probably evaluate the effect of nicotine on this endocrine gland.

To date, as indicated in accompanying progress reports etc., acute administration of nicotine to the spontaneously hypertensive rats stimulated adrenocortical (corticosterone) and adrenomedullary (catecholamine) release along with mobilization of FFA and increased glucose and cholesterol levels responses probably due to increased catecholamine output. One questions whether the significant depletion noted in K⁺ (76) levels by the larger dose at 30 minutes and both doses at the 1 hour interval may possibly be the result of a nicotine-induced release of mineralocorticoid hormones.

Evaluation of the effects of prolonged administration (6 weeks and 29 weeks) revealed in general no evidence of pronounced or restrained hypertensive effects on systolic blood pressures of the nicotine treated spontaneously hypertensive rats. In contrast, the SHR group showed consistent trends of hypotensive effects which at times were statistically significant during the 29 week oral administration period.

In general, oral nicotine administration showed pronounced decreases in the body weights of the treated spontaneously hypertensive and normotensive rats.

1003541960

Item 9 (continued)

Biological Testing of the Nicotine Analogs

I have been in communication with Professor Holger Erdtman who is also interested in the problem of nicotine activity. He and his colleague Professor U. S. v. Euler at the Karolinska Institute, Stockholm, Sweden, are eager to examine the stereochemically pure methyl-nicotines which we propose to make. The synthesis of additional analogs will depend on the pharmacological results obtained by v. Euler. The compounds are tested in the following biological systems using 1-nicotine as a standard.

1. Isolated rabbits jejunum
2. The guinea-pig ileum
3. The blood pressure of the cat
4. The isolated rectus abdominus muscle of the frog Rana temporaria

1003542085

in brain tissue.

The use of specific inhibitors and releasors for specific storage granules substrate system, as we have previously outlined, will serve as a basis for the isolation of such substrates from the specific tissue sites considered as a consequence of stress or in interaction with nicotine treatment. An interesting observation may be made in that most of those tissue and storage granule systems to which reference has been made, there are endogenous variations in both substrate levels as well as in ratios of "free" and "bound" substrates. This consideration will be given attention in several experiments specifically designed to consider cyclical variations in substrate content and storage and how this may provide a basis for differences in the effects of stress and/or nicotine treatment.

The means by which and rationale for isolation and selection of specific subcellular components from those indicated tissue sites under stress or in combination with nicotine treatment have been previously outlined; the significance of this approach lies in the ability to specify changes in the subcellular distribution of substrates, which by virtue of either stress and/or nicotine treatment may be changed through alterations in a "bound" to "free" disposition of a given substrate. This approach will also provide a source for relative purified subcellular components which can be isolated either following in vivo studies and separated for in vitro studies in order to investigate the potential difference in the response of such organelles originating from different cell sources to exogenous substrates comparable to those contained within these cellular storage sites.

(5) Stress-Nicotine Interactions and Central Nervous System Models.

In the central nervous system, it has been generally agreed that the most vulnerable component of change, pathological processes, insult, or phasic event

1003542042

PUBLICATIONS

- Nelsen, Judith M. and Conan Kornetsky: Single Dose Tolerance to Morphine Sulfate: EEG Changes. The Pharmacologist 10: No. 2, 1968.
- Weil, Andrew T., Norman E. Zinberg, and Judith M. Nelsen: Clinical and Psychological Effects of Marihuana in Man. Science 162: 1234-1242, 1968.
- Nelsen, Judith M. and Leonide Goldstein: Improvement of Performance on an Attention Task with Chronic Nicotine Treatment in Rats. The Pharmacologist 13: No. 2, 1971.
- Nelsen, Judith M. and Leonide Goldstein: Improvement of Performance on an Attention Task with Chronic Nicotine Treatment in Rats. Psychopharmacologia 26: 347-360, 1972.
- Nelsen, Judith M. and Conan Kornetsky: Morphine-Induced EEG Changes in Central Motivational Systems: Evidence for Single-Dose Tolerance. Fifth International Congress on Pharmacology (Abstracts, p. 166, #993), 1972.
- Goldstein, Leonide and Judith M. Nelsen: Some Views on the Neurophysiological and Neuropharmacological Mechanisms of Storage and Retrieval of Information. In: Memory and Transfer of Information (H.P. Zippel, ed.). Plenum Press, New York, 1973, pp. 155-191.
- Nelsen, Judith M.: Neurophysiological and Behavioral Consequences of Chronic Nicotine Treatment. In: Drug Addiction, vol. III (J.M. Singh, L.H. Miller, H. Lal, eds.). Futura Publishing Co., Mount Kisco (N.Y.), 1973 (Chapter accepted for publication).
- Nelsen, Judith M. and Leonide Goldstein: Chronic Nicotine Treatment in Rats: 1. Acquisition and Performance of an Attention Task. Res. Comm. Chem. Pathol. and Pharmacol. 5: 681-693, 1973.
- Nelsen, Judith M., Kathleen Pelley, and Leonide Goldstein: Chronic Nicotine Treatment in Rats: 2. Electroencephalographic Amplitude and Variability Changes Occurring Within and Between Structures. Res. Comm. Chem. Pathol. and Pharmacol. 5: 694-704, 1973.

1003542068

10. Space and facilities available (when elsewhere than item 2 indicates, state location). The unit is comprized of 4 specially designed rooms, 2 of which are 10.5' x 16.5' and 2 are 10' x 10'. There are also 3 offices, 10.5' x 12.5'. One of the larger laboratory rooms is equiped for behavioral measurements while the other larger laboratory is equiped for electroencephalographic recordings. All the necessary equipment for this proposal is available largely purchased with funds previously allocated by the Council.

Animal care and maintenance is provided on a per diem basis at the Vivarium of the Medical School, a short distance away. There is an underground tunnel between the 2 buildings. A full time Veterinarian is present.

A part-time secretary is available. We have a Model 1766 Monroe automatic Desk Computer with a card reader and programs for most computations needed for the project.

11. Additional facilities required As mentioned above these are available.

12. Biographical sketches of investigator(s) and other professional personnel (append).

13. Publications (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

See enclosed list. Reprints of 2 articles are enclosed. More will be sent as soon as they are available.

1003542055

14. First year budget:

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

	% time	Amount
Edward F. Domino	20	0
Theodore Spaulding	100	10,000

Technical

Michael Lutz, Laboratory Technician	100	9,000
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Secretary (to be recruited)	33	2,500
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Sub-Total for A \$21,500

B. Consumable supplies (by major categories)

Animals	\$2,500
Surgical	1,000
Chemical	2,000
Behavioral	2,000
Gas chromatographic	1,000

Sub-Total for B \$ 8,500

C. Other expenses (itemize)

Travel to yearly pharmacology \$ 700
meetings for professional personnel

12% Staff benefits: 2,580

Repair contract for scintillation counter	750	Sub-Total for C	<u>\$ 4,030</u>
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Running Total of A + B + C \$34,030

D. Permanent equipment (itemize)

Behavioral Programming Equipment	\$5,000
Photoelectric Motor Activity (Motron Productor)	3,000
Rat Stereotaxic	750
Gas Chromatographic Peak Integrator	5,000

Sub-Total for D \$13,750

E. Indirect costs (15% of A+B+C)

E 5,105

Total request \$52,885

15. Estimated future requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	21,500	8,500	4,030		5,105	\$39,135
Year 3	21,500	8,500	4,030		5,105	\$39,135

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In a comprehensive study concerned with the effects of both acute (restraint) and chronic stress (isolation) considerable data have been accumulated for inter-relating such variables as serotonin turnover, histamine content, gastric pH, etc., to the emergence of gastric tissue pathology. The relative contributions of these amines and their respective storage sites within the gastrointestinal system will be considered, specifically with reference to their role in response to stress as well as to nicotine treatment and the conditions under which these two events participate either temporally or spatially in the interaction with one another.

(2) Aside from those basic schema for acute and chronic stress specified above, several additional conditions will be imposed which have been shown to serve as effective models, particularly for somatic changes. These include (a) footshock, (b) electroconvulsive shock, and (c) sleep deprivation. The means by which these stressors are effected and the parameters utilized in their initiation have been worked out and lend themselves easily to use and adaptation in our laboratory.

The areas of primary attention toward which the above tissue systems will be directed will concern the relationship between "free" and "stored" pools of the specific tissue amine under consideration and the quantitative relationship between those tissues wherein multiple amine-regulated function is indicated. It is therefore our purpose to consider, on the basis of molecular ratios, those changes brought about by specific stressors in amine storage systems which provide for a release from storage in another amine system. An example illustrating this relationship may be found in adrenal tissue wherein adrenaline is released from chromaffin granules by the appropriate stimulation through nerve endings which release acetylcholine.

It is surprising, however, that these requirements for such adrenergic release have never been specified in terms of qualitative synaptic release of acetylcholine. Similar conditions prevail in cardiac tissue as well as, of course,

1003542041

Item #7. Brief Description of Specific Research Aims

As indicated in the progress report, both the test SHR and NR groups revealed significant increases in the relative adrenal weights (29 week study). No consistent findings were observed in the other relative organ weight analyses.

It is evident that the test spontaneously hypertensive rats (6 and 29 week studies) showed significant decreases in plasma cholesterol levels but no comparable alterations in plasma FFA titers. A trend of similar decreases in the cholesterol levels of the test normotensive rats after 29 weeks of treatment was not significant. After 29 weeks, significant decreases were observed in the plasma glucose levels of the nicotine treated normotensive rats but smaller decreases in the SHR group were not statistically significant. One questions the possible differential effects of nicotine on the regulation of adrenocortical, adrenomedullary and insulin secretory processes in the spontaneously hypertensive and normotensive rats.

The following investigation therefore has several continuing objectives:

1. To further study possible differential effects of prolonged administration of nicotine on systolic blood pressure responses of spontaneously hypertensive and normotensive rats.

2. By various biochemical, organ weight and histological procedures to evaluate differential effects of nicotine on adrenocortical (glucocorticoid and mineralocorticoid) adrenomedullary (catecholamine), gonadal (17-ketosteroid etc.), and the pancreatic hormonal systems of the hypertensive and normotensive rats and their relationships to the possible development of hypertension and pathology.

3. As a result of significant decreases in the plasma cholesterol levels of the spontaneously hypertensive rats, to initiate a complete blood lipid profile study of the effects of nicotine in the SHR and normotensive strains. This would include plasma cholesterol (free and total), plasma FFA, triglyceride and phospholipid levels in addition to evaluating the comparative effects of nicotine on the amounts of stored body fats. In view of the oft-cited relationship of high blood cholesterol and lipid levels to the development of hypertension and atherosclerosis, etc., this aspect should be of significant import.

4. An additional continuing aim is to determine via macroscopic and histological observations the gradual etiological and progressive development of cardiovascular and related pathologies in the spontaneously hypertensive and normotensive rats either related to age or administration of nicotine.

The present investigators have published investigations with hallucinogens such as lysergic acid diethylamide (45-49) and mescaline (50-54) on the metabolism behavior and endocrine function of rats and mice.

Our Laboratory has also engaged in studies related to the effects of auditory stress (55-57), vibration stress (58-60), isolation stress (61-65) as well as behavioral, metabolic and physiological differences in audiogenic-seizure susceptible vs. resistant rats (66-68) and the excitable homozygous-whirler vs. normal, heterozygous-whirler mutant mice (69-75). The various behavioral, biochemical and endocrine studies have indicated heightened metabolism rates, increased adrenocortical function and, in general, inhibited gonadal activity in the whirler mice. These may be symptomatic and correlated with physiological and neuronal changes responsible for the wild, circling, locomotor activity. Biochemical alterations have indicated significantly increased plasma corticosterone (72,73), adrenal corticosterone (72, 73) and adrenal catecholamine levels (73)

1003541961

Item 13 (continued)

Recent publications (reprints are attached at the end of this application)

- E. Leete, M. R. Chedekel, and G. B. Bodem Synthesis of Myosmine and Normicotine, Using an Acyl Carbanion Equivalent as an Intermediate J. Organic Chem., 37, 4465 (1972).
- E. Leete, Biomimetic Synthesis of Nicotine. J. Chem. Soc., Chem. Commun., 1091 (1972).
- E. Leete and A. R. Pinder, Biosynthesis of Dioscorine, Phytochemistry, 11, 3219 (1972).
- E. Leete and M. R. Chedekel, The Aberrant Formation of (-)-N-Methyl-Anabasine from N-Methyl- Δ^1 -piperideinium Chloride in Nicotiana tabacum and N. glauca, Phytochemistry, 11, 2751 (1972).
- E. Leete and J. O. Olson, Biosynthesis and Metabolism of the Hemlock Alkaloids, J. Amer. Chem. Soc., 94, 5472 (1972).
- E. Leete, Chapter 5 in "Biosynthesis" , A specialist Periodical Report of the Chemical Society, London, Edited by T. A. Geissman, 1972 , Biosynthesis of Alkaloids pp. 158-240.

1003542088

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

August 1, 1973

Grant Application No. 929
PHARMACOLOGY

To: The committee comprising Drs. Gardner, Jacobson, and
Sommers

Subject: Edward Leete, Ph.D., University of Minnesota
New application No. 929
"Synthesis and Biological Activity of Nicotine
Analogues"

History

In 1969 Leete's application for support of a study
"Effect of External Factors on Metabolism in the Tobacco Plant"
was denied.

The present proposal was Case No. 223 and application
was encouraged.

Application No. 929 requests \$23,709 plus two addi-
tional years.

Documents Submitted

Attached is application dated 7.25.73.

Reprints of the recent publications by Leete et al.
listed on page 3c of the application were provided and will be
forwarded if you so request.


F.W.N.

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Encl.

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In Table 7, data for in vitro prove REFERENCES by in vivo experiments are summarized.

1. Caputo, D.V., Essman, W.B., Teitler, R., Loewe, G., & Frisone, J.D.:

Housing modification as a variable in fasting-induced ulcerogenesis.

J. Psychosom. Res., 1968, 12, 129-135.

2. Essman, W.B.: Gastric ulceration in differentially housed mice. Psychol.

Rep., 1966a, 19, 173-174.

3. Essman, W.B.: Isolation-induced facilitation of gastric ulcerogenesis in

mice. J. Psychosom. Res., 1966b, 10, 183-188.

4. Essman, W.B.: The development of activity differences in isolated and

aggregated mice. Anim. Behav., 1966c, 14, 406-409.

5. Essman, W.B.: Differences in locomotor activity and brain serotonin

metabolism in differentially housed mice. J. Comp. Physiol. Psychol.

1968, 66, 244-246.

6. Essman, W.B.: "Free" and motivated behavior and amine metabolism in isolated

mice. In: Garattini, S., & Sigg, E.E. (Eds.). Aggressive Behaviour.

Amsterdam: Excerpta Medica, 1969, Pp. 203-208.

7. Essman, W.B.: Isolation-induced behavioral modification: Some neurochemical

correlates. In: Serman, M.V., McGinty, D.J. & Adinolfi, A.M. (Eds.).

Neural Ontogeny and Behavior, N.Y.: Acad. Press, 1970a, (In Press)

8. Essman, W.B.: Differential housing in mice: a source of behavioral and neuro-

chemical change. In: Riesen, A.H. (Ed.). Maternal-social deprivation

as a functional somatosensory deafferentation in the abnormal development

of the brain and behavior. Washington, D.C.: Amer. Psychol. Assn., 1970b,
(In Press).

9. Essman, W.B.: Neurochemical changes associated with isolation and environmental

stimulation. In: J. Biol. Psychiat., 1970c, (In Press).

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#909-ROSECRANS

1003542102

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The chemistry department at the University of Minnesota has well equipped laboratories for all types of chemical research. All modern instruments are available to aid in elucidation of structures, especially the stereochemistry of organic molecules. The equipment available to the principal investigator includes the following: MS-30 mass spectrometer with computer output, Varian XL-100 NMR spectrometer, Nuclear Chicago Mark II liquid scintillation counter, several IR, UV and ORD (Cary 60) spectrophotometers, several gas chromatograms, a Waters high pressure liquid chromatography system. The principal investigator has an active research group (currently 10 graduate students and one postdoctorate fellow). Most of this group is housed in a new building which was opened in April 1971.

11. Additional facilities required:

None

1003542086

12. Biographical sketches of investigator(s) and other professional personnel (append):

Edward Leete (principal investigator), George B. Bodem {graduate student}
Philip Hoekstra {graduate student}

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

In Table 7, data for in vitro protein synthesis by liver microsomes are summarized and these data confirmed previous observations indicating an increase in such synthesis as a consequence of chronic stress.

Brain protein synthesis was significantly altered in the myelin fraction from the cerebral cortex of chronically stressed mice as shown in Table 8. It should be pointed out that these observations are merely preliminary and considerably more data should be outlined before any more definite conclusions can be reached. It is clear, however, that morphological, biochemical, and cellular changes are associated with stress and may be utilized as indices in assessing such stress effects. The effects of nicotine treatment both per se, as well as in interaction with acute and chronic stress would appear ideally to constitute a relevant independent variable by which, particular cellular changes may be further studied. Inasmuch as the active constituents in tobacco smoke, aside from nicotine, warrant consideration as pharmacological variables, it would seem that those systems proposed constitute firm bases upon which such pharmacological evaluation may be approached.

1003542048

7. (a) Our previous application to The Council for support (dated October 20, 1972) outlined in some detail the specific aims of the proposed studies. However, it might be useful to review these aims in light of the experimental objectives which have already been achieved. On the basis of studies carried out under past grants from The Council, it was reported that following chronic nicotine treatment there occur certain changes in the features of the electrophysiological mechanisms of arousal. These changes were interpreted as being indicative of a shift from the "classical" arousal mechanism [involving the mesencephalic reticular formation (RF)] to arousal mediated by the limbic system (Bhattacharya and Goldstein, Neuropharmacol. 9:109-118, 1970). We recently completed and reported a study of the effects of chronic nicotine administration on the electrical activity within and between brain structures of Sprague-Dawley rats which generally confirmed the earlier findings in rabbits (Nelsen, Pelley, and Goldstein, Res. Comm. Chem. Pathol. and Pharmacol. 5:694-704, 1973).

The proposed consequence of the electrophysiological changes was that behavior should become more specifically goal-oriented since, as Routtenberg suggested (Psychol. Rev. 75:51-80, 1968), the main functional significance of predominant limbic system mediation involves "incentive-oriented" arousal while that of the RF is "drive-oriented". Working under subsequent grants from The Council, we tested this proposal in rats rigorously trained so that they performed

1003542058

6. (Continued...)

Throughout the course of the present and past studies in this laboratory, the aim has been to provide basic biochemical and pharmacological information, which will aid in understanding the biological and psychological implications of exposure to nicotine and its congeners. Consistent with that point of view studies on nicotine and its metabolites (natural and synthetic) have continued in this laboratory.

During the past year other laboratories upon their request have been supplied with samples of nicotine metabolites at an average rate of one sample every two weeks. Unfortunately some requests were denied because of paucity of material. It is believed that this service to others has provided both parties of the transactions a better appreciation of the scientific problems.

1003542097

9. (b) cortical effects of D-amphetamine, methyl phenidate, caffeine, physostigmine, pemoline, L.S.D., and tetrahydrocannabinol in animals treated chronically with either nicotine or saline will be made.

Electric current will be delivered by a pair of Grass stimulators which have been modified to obtain a reliable "constant current" output. Parameters of the current will be within a moderate range which has been shown to cause only reversible effects both on behavior and neural tissue (Kornetsky and Eliasson, 1969; Nelsen, 1970).

The second area of investigation involves behavioral measures of the effects of modifications in the proposed balance between limbic and RF control of arousal. Because in our hands it has proven to be impressively sensitive to levels of arousal, the same form of the behavioral task of Kornetsky and Elisson (1969) which we have described in previous grant applications to the Council (dated November 25, 1970 and October 25, 1971) will be used to assess the functional consequences of electrical and pharmacological manipulations of the RF arousal pathway.

Twelve rats will be prepared surgically with electrodes at the sensory-motor cortex and in the mesencephalic RF. Following recovery (about three weeks), these animals will be trained to perform on the visual attention task. The rats will be partially food deprived and maintained at approximately 85% of their normal, free-feeding body weights. They will be trained to press a lever for a food pellet reinforcement

1003542062

Supporting data and indications of project feasibility.

The appended grant progress report represents a summary of thirty-seven key experiments wherein data in support of chronic stress or chronic-acute stress interactions have been considered in view of physiological, pharmacological, neurochemical and behavioral changes observed in our laboratory with one species of mouse. There are in addition, several pending publications which have been appended to this application; these bear further upon some of the evidence cited in sections of the foregoing proposal and provide considerable support to warrant the use of both the techniques outlined as well as the experimental parameters.

Additionally, recent supporting data from our laboratory has been summarized. In Tables 1 and 2, the effects of chronic stress (isolation) are indicated with regard to the hepatic microsomal metabolism of pentobarbital and pentobarbital sleeping time; it may be observed from these tables that the stressed animals showed shorter barbiturate sleeping times and can be accounted for by an increased rate at which the drug metabolism enzyme is included in the liver. In further consideration of the differences in this regard, Table 3 illustrates the effects of chronic stress (isolation) on liver weights in mice as a function of the duration of such stress. It may be observed that as the duration of stress is increased, the liver weight is increased relative to total body weight, as compared with non-stressed animals.

In Table 4, it may be observed that increased chronic stress leads to increased in in vivo incorporation of amino acid into protein and from Table 5, it may be observed that microsomal ATPase activity is reduced both in liver and brain fractions as a consequence of isolation. Oxidative phosphorylation in liver mitochondria was generally not as great in magnitude (Table 6) as one might expect.

1003542047

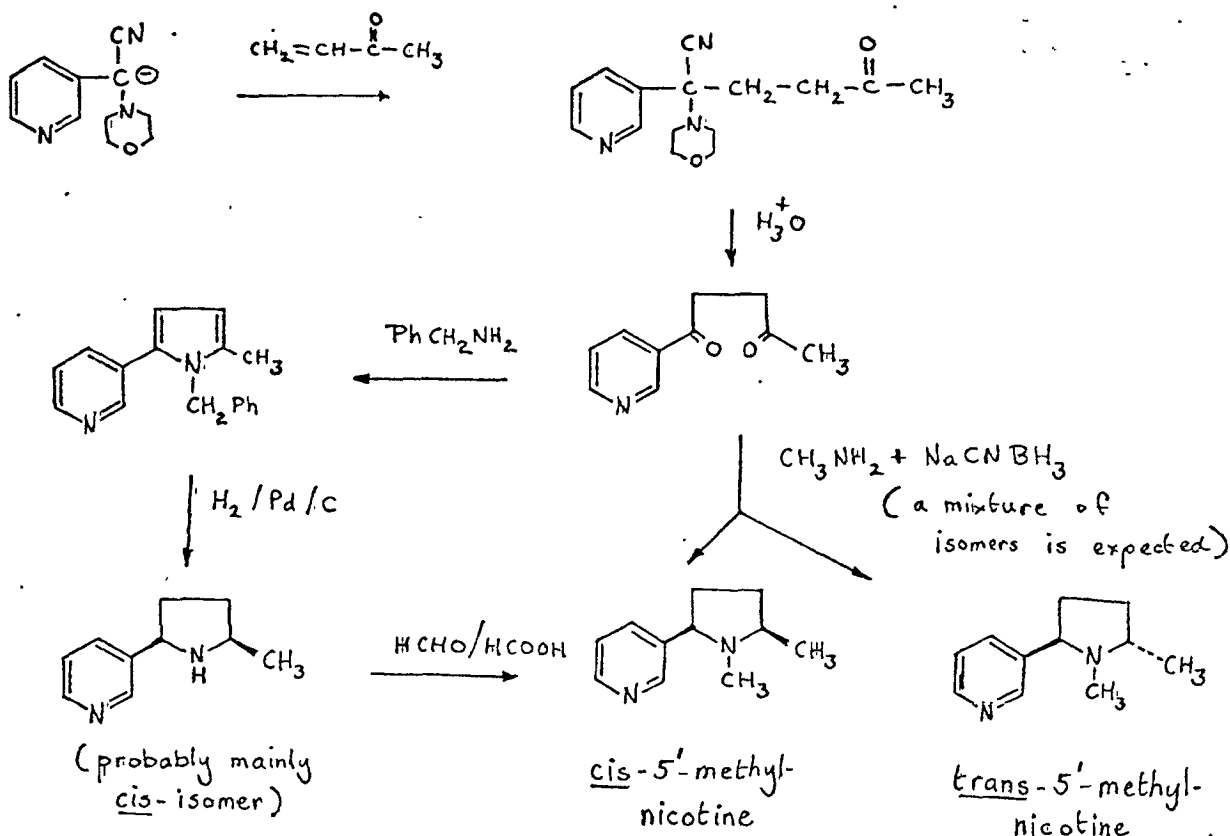
9. (c) following the presentation of a conditional stimulus (C.S.) which is the white cue light in a standard operant conditioning box. Training is carried out in a series of phases such that initially the task is quite elementary, i.e., continuous reinforcement or fixed ratio 1 in the presence of a constant C.S. During successive training sessions, an inter-trial interval (I.T.I.) is introduced and the duration of the C.S. is reduced in step-wise fashion while a punishment contingency for inappropriate responding is also added. In the task's final form, the duration of the cue light is only 0.2 sec, followed by an available response time of 5.0 sec during which only the first lever press is reinforced. Failure to press after a C.S. is scored as an omission error (o.e.) and has no consequence for the animal other than the loss of a reinforcement pellet. The I.T.I. is variable (so that the rat is not learning to time responses according to a fixed interval) with a mean of 10.0 sec. A lever press during the I.T.I. is scored as a commission error (c.e.) and is punished by the imposition of a 30.0 sec "time-out". Additional responses during the time-out reset the punishment clock to 30.0 sec and are also scored as c.e.'s. A session is terminated after 100 reinforcements have been delivered or after one hour has elapsed. The task is programmed via electro-mechanical modules.

This type of task in which the animal is asked not only to make appropriate responses but also to inhibit inappropriate responses is difficult for rats to learn and requires several months of training to achieve efficient performance which we

Item 9 (continued)

5'-Methylnicotine

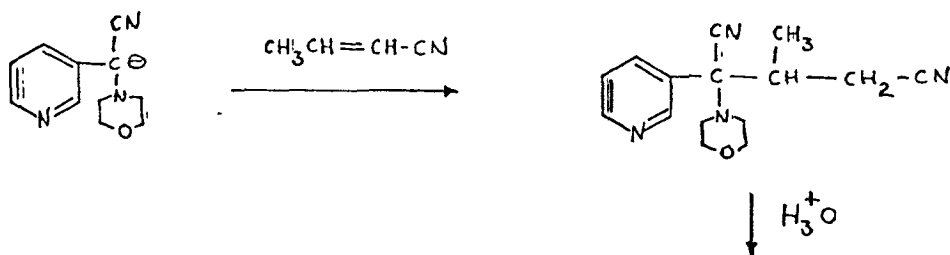
This compound has been described by I. Yamamoto (Agr. Biol. Chem. (Tokyo), 27, 445 (1963)), however the stereochemistry of the methyl group was not determined and presumably a mixture of isomers was obtained.



The 5'-dimethylnicotine has been prepared by Castagnoli (J. Pharm. Sci., 58, 860 (1969)) and A. Burger (J. Pharm. Sci., 59, 342 (1970)).

3'-Methylnicotine

The trans-isomer of this compound has been prepared by Castagnoli (J. Org. Chem., 37, 1268 (1972)). It is expected that the following method will furnish both the cis and the trans isomers.



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#869R1 - RUBIN

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THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

July 6, 1973

Grant application No. 917

TO: The committee comprising Drs. Ding, Gardner, Meier

SUBJECT: Patricia M. Hudgins, Ph.D., Medical College of Virginia, Richmond
New application No. 917
"Possible synergistic sympathomimetic actions of nicotine and
acetaldehyde on the cardiovascular system"

History

This is a spontaneous application, with no known antecedents.
Application #917 requests \$22,400 plus two additional years.

Documents Submitted

Attached is application dated July 1, 1973.

Reprints of the five papers listed under item 13 on page 3a have
been provided, and will be forwarded if you request.

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F.W.N.

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is free to exert its pharmacological actions and can be taken up at amine storage sites where it is not normally present.

Isolation of platelets will be accomplished by sedimentation and differential centrifugation, allowing for both platelet counts as well as the study, in vitro of these units; serotonin content, precursor uptake and catabolism will be considered during stress and with the conditions wherein nicotine is also utilized as an independent variable. Direct extraction and reading of serotonin from platelets permits rapid and reliable assay of extremely small quantities of this amine. The general experimental procedures to be followed parallel those described previously.

(4) Gastrointestinal Tissue.

The role of 5-hydroxytryptamine (serotonin) in the regulation of the mechanical action of the intestine appears to reside in its stimulation of receptors in the mucosal tissue, as well as by providing sensitization to endogenous choline. The release of serotonin from its gastrointestinal storage sites, has been accomplished by a variety of stimuli including stress. Preliminary investigation of the relationship between acute stress (restraint) and gastric ulcer production in mice has led to the implication of serotonin in this process (Essman, et. al., 1971). Histamine, peculiar to its own storage granules in the gastrointestinal system (the mast cell) has also been implicated as being important for stress; as both a causative factor, as well as a potential correlate by way of enzyme systems in common with gastrointestinal serotonin. The basis upon which present consideration of these two important biologically active amines is considered relates to their potential value as site-specific molecules related to both stress and nicotine treatment and being further able to describe the interaction of these two events.

1003542040

be reversed (7) Oxytremorine is a substance specific to the cholinergic system, wherein its effect is to produce a significant elevation in tissue acetylcholine, the duration of which is dose-dependent. This substance may thereby be utilized to produce a reversible increase in cholinergic content of cardiac tissue, wherein its major effects following parenteral administration may be observed.

(8) Methyl Atropine is a compound which is specific to peripheral inhibition of post ganglionic cholinergic nerves and a blockade of the muscarinic effects of acetylcholine. There is considerable evidence to indicate that acetylcholine storage is modified by atropine-like compounds which lead to the depletion of this amine. It would therefore be appropriate to consider reduced cholinergic function brought about by this drug in cardiac tissue.

(1) Cardiac Tissue.

Catecholamines (epinephrine, norepinephrine) have been studied extensively in cardiac tissue. There seems to be a divergence of opinion as to whether or not the catecholamine containing structures are the cell bodies of this tissue or adrenergic nerve terminals. Catecholamine containing cells have been described (Jacobowitz, et. al., 1966), however these observations have not been confirmed by others (Dahlstrom, et. al., 1965). Within the neurons innervating cardiac tissue, there is evidence for different storage pools for norepinephrine. Specific micro-particles (20-100m μ) have been isolated from cardiac tissue (Michaelson, et. al., 1964) in which a large fraction of the cardiac norepinephrine is bound. This apparently is represented in a partial fractionation of cardiac homogenates on the order of approximately 60-80% of the total cardiac content of this amine (Glassman, et. al., 1965). The presence of "free" norepinephrine apparently remains in considerable question. Histochemical evidence strongly suggesting the absence of any extraneuronal pool of this amine in cardiac tissue. Unfortunately this question

1003542034

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

August 3, 1973

Grant Application No. 868R1
PHARMACOLOGY

To: The committee comprising Drs. Bing, Gardner, and Jacobson

Subject: Herbert McKennis, Jr., Ph.D., Medical College of Virginia
First renewal No. 868R1
"Biological Activity of Tobacco Smoke Components and Allied Substances"

History

CTR has supported various studies by this applicant since 1960.

Current Grant No. 868 is for \$60,000. One additional year priority was voted at an amount not to exceed \$60,000.

Application No. 868R1 requests \$74,184. This amount is defended in Dr. McKennis's letter to Dr. Hockett dated July 27, copy appended.

Documents Submitted

Attached is application dated July 27, 1973 (7 pages).

Also attached is Progress Report No. 1, October 1, 1972 - July 30, 1973 (27 pages).

Copies of recent abstracts and manuscripts were provided, and will be forwarded if you wish.

Comment

A site visit is planned before the October meeting.

F.W.N.
F.W.N.

FWH:wg
Encls.

1003542092

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

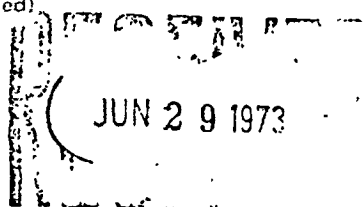
110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application for Research Grant
(Use extra pages as needed)

Date: July 1, 1973

1. Principal Investigator (give title and degrees):

Patricia M. Hudgins, Ph.D.
Associate Professor



2. Institution & address:

Medical College of Virginia, Health Sciences Division,
Virginia Commonwealth University, Richmond, Virginia 23298

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

Possible synergistic sympathomimetic actions of nicotine and acetaldehyde
on the cardiovascular system.

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: Three years

7. Brief description of specific research aims:

Specific Aim 1. To compare and contrast the cardiovascular actions and interactions between intravenous nicotine, acetaldehyde and tyramine in the anesthetized rat. Various surgical and pharmacologic procedures will be used to establish the precise mode of cardiovascular action of nicotine, acetaldehyde and tyramine. Combinations of these agents will then be used to test for potential interactions producing additive or synergistic cardiovascular effects. The ability of single agents to reverse the hypotensive effect of guanethidine pretreatment will be compared to combinations of the sympathomimetic agents.

Specific Aim 2. To examine the actions and interactions of nicotine, acetaldehyde and tyramine at the cellular level in smooth muscle preparations in vitro. Perfused central ear artery and aortic strips from rabbits and isolated rat vas deferens preparations will be used to compare sympathomimetic actions of nicotine, acetaldehyde and tyramine. Sympathetic nerve function will be altered in these tissues by guanethidine, tetrodotoxin and calcium deprivation in order to examine the effect on contractile responses evoked by transmural stimulation and the sympathomimetic agents. The actions and interactions between these drugs and ^{14}C -norepinephrine will be studied to confirm the role of transmitter release in drug-induced tissue responses to single agents and combinations of nicotine and acetaldehyde.

1003542071

Comm

CARDIOVASCULAR

Dr. Bing
Dr. Jacobson
Dr. Sommers

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

Application for Research Grant
(Use extra pages as needed)

Date 7-17-73

1. Principal Investigator (give title and degrees).

A. Stanley Weltman, Ph.D., Associate Professor in Pharmacology and Research

2. Institution & address

Laboratories for Therapeutic Research
Research Institute of the Brooklyn College of Pharmacy
Brooklyn College of Pharmacy
Long Island University
600 Lafayette Avenue Brooklyn, N. Y. 11216

3. Department(s) where research will be done or collaboration provided

- a) Laboratories for Therapeutic Research
- b) Institute of Pathology, Downstate Medical Center, S. U. M. Y.
Brooklyn, N. Y.

4. Short title of study

Effects of Nicotine on Blood Pressure, Blood Lipid Profile, Endocrine
Activities and Pathology of Spontaneously Hypertensive and Normotensive Rats

5. Proposed starting date.

January 1, 1974

6. Estimated time to complete.

two years

7. Brief description of specific research aims:

The proposed investigation is being submitted to continue our previous research, "Acute and Chronic Effects of Nicotine and Pathology in Spontaneously Hypertensive and Normotensive Male Rats," awarded under CTR Grants 833; 833R1. Furthermore, an additional goal of the investigation is a detailed study of the plasma lipid profile (cholesterol, FFA, triglycerides, phospholipids) in test and control spontaneously hypertensive and normotensive rats. Initially, the investigation sought to determine possible synergistic and cumulative hypertensive or hypotensive effects contributed by acute subcutaneous and chronic (oral) administration of nicotine to a genetically selected strain of spontaneously hypertensive rats (SHR) and a normotensive strain of Wistar rats (NR). It was anticipated that the study of various biochemical, physiological and morphological differences in treated and untreated hypertensive and normotensive animals sacrificed at various age levels would contribute further knowledge of plasma cholesterol, FFA, Na^+ , K^+ and glucose metabolism and regulation as well as evidence of endocrine relationships to hypertension. Consequently, biochemical evaluations have included plasma corticosterone, adrenal corticosterone, adrenal catecholamines (epinephrine, norepinephrine and total catecholamines), plasma glucose, FFA, total plasma proteins, plasma Na^+ and K^+ levels and urine assays of 17-ketosteroid titers (androgens).

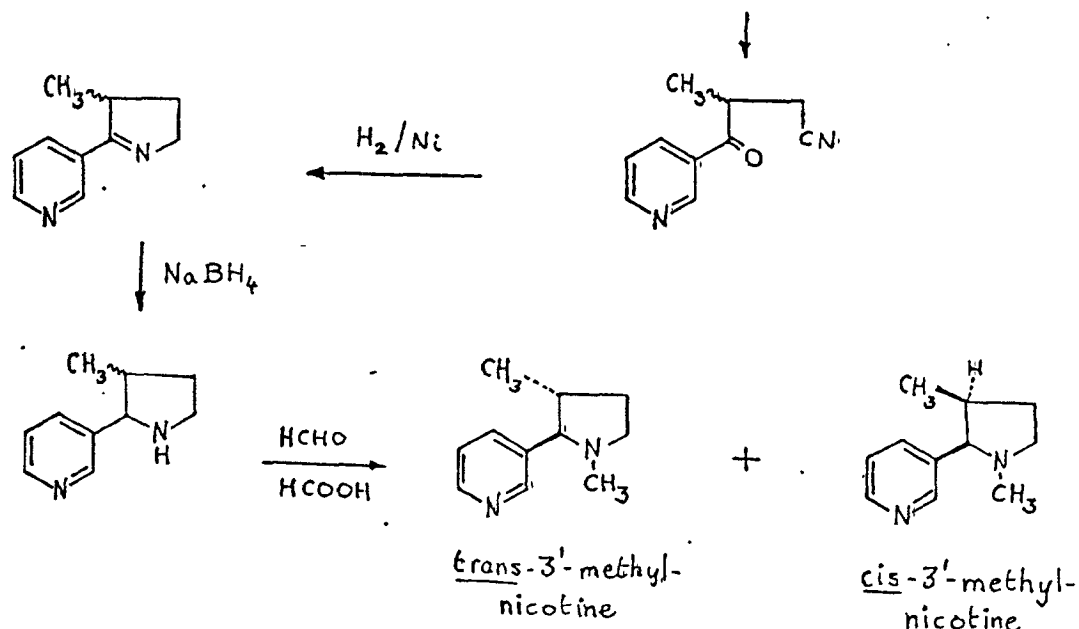
An additional objective was and is to determine via detailed macroscopic and histological examinations the gradual etiological and progressive development of cardiovascular and related pathologies in the spontaneously hypertensive

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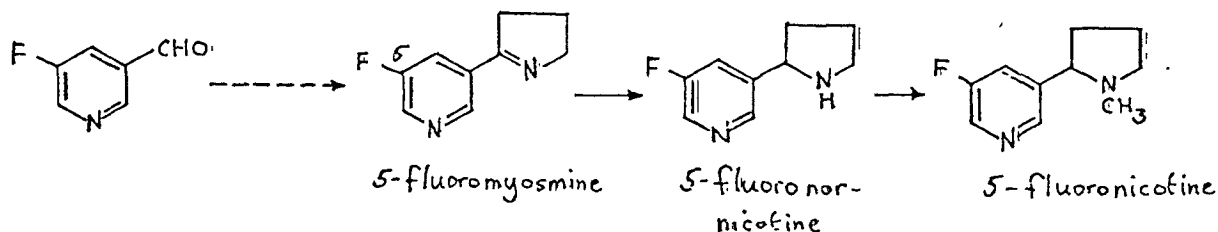
In general, the term of these processes is REFERENCES

1. Baumgartner, H.R., & Born, D.V.R. (1968). Nature (London) 218: 137-141.
2. Blaschko, H. (1954). Pharmacol. Rev., 6: 23.
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9. Elmadjian, F., Lamson, E.T., & Neri, R. (1956). J. Clin. Endocrinol. Metabol., 16: 222.
10. Elmadjian, F., Hope, M.H., & Lamson, E.T. (1957). J. Clin. Endocrinol. Metabol., 17: 608.
11. Essman, W.B., Essman, S.G., & Golod, M.I. (1971). Pyhsiol. Behav. (In Press).
12. Glassman, P.M., Angelakos, E.T., & McNary, W.F. (1965). Life Sci., 4: 1727.
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15. Johnson, S.A., Monto, R.W., Rebuck, J.W., & Horn, R.C., Jr. (Eds.). (1961). Blood Platelets, Little Brown and Company, Boston.
16. Kowalski, E., & Niewiarowski, S. (Eds.). (1967). Biochemistry of Blood Platelets Academic Press, New York.
17. Kuske, H.J. (1961). Arch. Kreislaufforsch. 36: 104.

Item 9 (continued)

Other Nicotine Analogs

By the use of derivatives of pyridine-3-aldehyde in the above synthetic sequences it will be possible to prepare nicotine analogs with substitution in the pyridine ring. For example by the use of 5-fluoropyridine-3-aldehyde 5-fluoro derivatives of myosmine, normicotine, and nicotine will be prepared :



The fluorine group, being strongly electron attracting would be expected to reduce the basic strength of the pyridine nitrogen. This may have a profound effect on the binding properties of nicotine to the receptor site and its biological properties. We have prepared (RS)-5-fluoronicotine by another route (E. Leete, M. F. Manuel, and G. B. Bodem, *Phytochemistry*, 10, 2687 (1971)) and this analog is currently being tested by Von Euler.

1003542084

5

10. Outline of experimental protocol for the coming year.

Various aspects of the experimental protocol for the coming year are mentioned in Section 12 (Summary Progress Report). By and large the biological techniques are standard, including those employed in the study of peripheral vascular resistance (Konzett, Bost, Bowman, Bowman and McKennis, J. Pharmacol. Exp. Therap. 178, 122 (1971)). It will be noted in the Summary Progress Report that preliminary studies on the determination of nicotine and metabolites by mass fragmentography have been conducted during the past period. It is hoped that during the coming year improvements in these techniques can be developed. Design changes in commercial mass spectrographic apparatus may eventually bring mass spectrographic determinations to a level of sensitivity that will be useful in confirming other sensitive analytical procedures, including radioimmunoassays. Quick and reliable assay procedures, many of which are now lacking, are certainly desirable in many biological studies.

1003542099

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

June 20, 1973

Grant Application No. 909

To: The committee comprising Drs. Gardner, Meier, and Sommers
Re: John A. Rosecrans, Ph.D., Medical College of Virginia,
Richmond
New application No. 909
"State Dependent Properties of Nicotine Related Compounds"

History

This proposal originated as Case No. 142, and the then Planning Committee encouraged formal application.

Application No. 909 requests \$20,260 plus two additional years.

Documents Submitted

Attached is application dated 5/7/73.

Reprints of publications #1, 4, and 6 listed on page 10 have been provided, and will be forwarded on request.

Comment

Attached is an opinion we have obtained from Dr. Donald A. Overton.


F.W.N.

FWN:wg
Encls.

1003542103

#467C-WESTFALL

1003542142

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
"Studies of Nicotine Action on Memory Consoli- dation"	Council for Tobacco Research U.S.A.	\$21,579.	January, 1973 to December, 1973

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
"Studies of Nicotine Action on Memory Consoli- dation"	Council for Tobacco Research, U.S.A.		January, 1974 to December, 1974

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to:

Research Foundation

Mailing address for check:

1411 Broadway

New York, N.Y. 10011

Principal investigator:

Typed Name Walter B. Essman

Signature Walter B. Essman Date July 26, 1973

Telephone 212 762-5949 ---
Area Code Number Extension

Responsible officer of institution:

1) Hannah Petzenbaum

Typed Name 2) Albert M. Levenson

1) Research Foundation

Title 2) Assoc. Dean of Faculty - Queens College

Signature Hannah Petzenbaum Date 7/26/73

Telephone 212 445-5275 ---
Area Code Number Extension

1003542027

which would either block nicotinic or muscarinic receptors in the CNS, or would inhibit specific enzyme systems involved with the control of specific biogenic amine systems.

Aside from using these techniques to study mechanisms by which nicotine may be producing its behavioral effects, an attempt was also made to determine the specificity of this drug effect. In other words, we attempted to determine what drugs if any, could produce a nicotine cue.

Interestingly, nicotine did not transfer to any of the drugs studied.

Drugs such as d-amphetamine, arecoline and LSD and lobeline were studied in this regard. The rationale for the current study will be based upon the above ideas that drugs producing similar behavioral effects will transfer, or generalize, to each other. This has been shown amongst several drugs such as hallucinogenic drugs. In rats trained to discriminate between LSD and saline, mescaline was interpreted or perceived by these animals as producing effects similar to LSD. On the other hand, LSD would not transfer to a drug such as d-amphetamine. Rats given d-amphetamine responded as if they perceived, or had been given saline.

In view of this, we hope to ask the following questions concerning analogs and metabolites of nicotine:

1. Does a compound similar in structure to nicotine, or a metabolite of nicotine, have state dependent effects of its own?
2. Can the nicotine state transfer to the compound in question? More specifically, will an animal trained to discriminate between nicotine and non-drug states perceive such compounds as producing effects similar to nicotine.
9. Details of experimental design and procedures (append extra pages as necessary)

1003542106

Rosecrans, J.A.: Brain area nicotine levels in male and female rats with different levels of spontaneous activity. *Neuropharmacol.* 11: 863, 1972.

Schechter, M.D. and J.A. Rosecrans: Nicotine as a discriminative cue in rats: inability of related drugs to produce a nicotine-like cueing effect. *Psychopharmacologia* 27: 379, 1972.

Schechter, M.D. and J.A. Rosecrans: D-amphetamine as a discriminative cue: drugs with similar stimulus properties. *European J. Pharmacol.* 21: 212, 1973.

Schechter, M.D. and J.A. Rosecrans: Atropine antagonism of arecoline-cued behavior in the rat. *Life Sciences* 11: 517, 1972.

Rosecrans, J.A., M.H. Goodloe, Jr., G.J. Bennett and Ira D. Hirschhorn: Morphine as a discriminative cue: effects of amine depletors and naloxone. *European J. Pharmacol.* 21: 252, 1973.

1003542122

CURRICULUM VITAE

Judith M. Nelsen, Ph.D.

REDACTED

BORN:

MARITAL STATUS:

REDACTED

- 1946-60 Primary and secondary studies, public schools of Town of Lake and City of Cudahy, Wisconsin
- 1960-63 Undergraduate studies, University of Wisconsin-Milwaukee (Letters and Science, Pharmacy)
- 1963 Laboratory assistantship in bacteriology (University of Wisconsin-Milwaukee)
- 1963-65 Undergraduate studies, University of Wisconsin-Madison (Pharmacy, Psychology)
- 1963-65 Research assistantship in physical chemistry (University of Wisconsin-Madison)
- 1964 Summer research assistantship in physical chemistry (from the U.S. Department of the Army at the University of Wisconsin-Madison)
- 1965 B.S. (HONORS) degree. University of Wisconsin. Madison, Wisconsin
- 1965-70 Graduate studies, Boston University School of Medicine, Division of Medical Sciences, Department of Pharmacology and Experimental Therapeutics (Major professor: Conan Kornetsky, Ph.D., Director, Laboratory of Behavioral Pharmacology)
- 1965-66 Graduate School Research Fellowship
- 1966-70 Public Health Service Research Fellowships (N.I.M.H.)
- 1970 Doctor of Philosophy degree. Boston University. Boston, Mass.
- 1970-72 Post-doctoral appointment. N.J. Bureau of Research in Neurology and Psychiatry. Box 1000, Princeton, N.J.
- 1973 Senior Scientist. Rutgers Medical School. Department of Psychiatry, Piscataway, N.J.

HONORS: Sophomore Honors (U.W.); Senior Honors (U.W.); Sigma Epsilon Sigma (U.W.); Rho Chi (U.W.); Phi Kappa Phi (U.W.); Sigma Xi (B.U.)

PROFESSIONAL SOCIETY MEMBERSHIPS:

REDACTED

1003542067

4.

14. First year budget:

A. Salaries (give names or state "to be recruited")

	% time	Amount
Professional (give % time of investigator(s) even if no salary requested)		
Edward Leete (one month summer salary)	100	
George B. Boden (research assistant)	100	
Philip Hoekstra (research assistant)	100	
Fringe Benefits on the above salaries (calculated at 16.1 %)		
Technical		

Sub-Total for A

B Consumable supplies (by major categories)

Chemicals (organic compounds, solvents etc.)	3500
Glassware	1500

Sub Total for B

C Other expenses (itemize)

Analytical Services (mass spectra NMR, CHN)	600
Page charges for journal articles	300
Travel to one domestic scientific meeting	300

Sub Total for C

Running Total of A + B + C

D Permanent equipment (itemize)

None

Sub Total for D

E Indirect costs (15% of A+B+C)

3066

Total request

15 Estimated future requirements

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2	5000	1200	0	3180		
Year 3		1200	0	3300		

1003542089

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

We have three laboratories available for this research. These laboratories are equipped with behavioral and chemical instrumentation sufficient to conduct any aspect of the research described herein. However, if this grant was awarded, additional behavioral equipment would be needed to take of the increased research load.

11. Additional facilities required:

None Needed

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available):

1003542114

attempt to overcome the effects of the antagonist. Thus, aside from studying the general antagonist effects of such drugs, drug competition can also be evaluated in such studies.

D. Studies Involving the Discriminative Properties of Drugs to be Evaluated
In This Project

Besides studying the effects of experimental drugs in the passive and active avoidance studies indicated above, we would also like to determine if any active CNS drug has discriminative cue properties of its own. These studies will be conducted in the same manner as the procedures described above for studying nicotine as a discriminative cue (C). Dose regimens will be determined by previous studies. Such studies will be conducted in the same manner as above, and will be conducted for a period of 5 to 8 weeks. It is felt that animals which do not learn to discriminate between such a compound and non-drug states in that period of time does not have sufficient state dependent effects to study any further. If a compound still appears to be interesting from an experimental point of view based on other studies conducted in parts A-D, then we will make a more intensive evaluation of the state dependent properties of the drug.

If a compound can be shown to act as a discriminative stimulus or cue, then a more complete psychopharmacological investigation of this drug will be conducted. The types of studies to be conducted under this condition will involve dose response, and time duration experiments. Compounds having such discriminative properties will also be studied from the point of view of mechanism of action. Thus, these compounds will be studied to determine if they stimulate muscarinic cholinergic receptors. An appropriate blocking drug, such as atropine and mecamylamine will be utilized for this purpose. If such compounds have specific qualities resembling cholinergic properties of nicotine, we will also expand these studies to include in evaluation of

1003542112

10. Space and facilities available (when elsewhere than item 2 indicates, state location): Office and laboratory space are provided in the Department of Pharmacology for the principal investigator, lab specialist and parttime employee. These consist of three adjoining rooms on the fourth floor of McGuire Hall. The laboratory contains standard supplies: chemicals, glassware, balances, drying oven and refrigerators. Major items of equipment available in the laboratory are the following: Picker Nuclear low background proportional gas flow counter, Mettler analytical balance, Corning AG-1b glass still, Corning model LD-3 demineralizer, Grass model 5 D polygraph with FT .03 transducers, Grass model 79 two channel polygraph with pressure transducers, Monroe rotary (2) and electronic calculator, Eberbach shaker, Vortex mixer, Muffler furnaces (2), Thermolyn hotplates (2), Bausch and Lomb spectronic 20 and International clinical centrifuge. Other items of major equipment available in the department include liquid scintillation counters (2), Perkin-Elmer atomic absorption spectrophotometer and International refrigerated preparatory centrifuge.

Animal care facilities are located on the fourth and fifth floors of McGuire Hall. These are staffed and maintained by departmental personnel. A fully-equipped machine shop is also located on the fourth floor. This shop is staffed with a machinist who designs and fabricates custom apparatus.

11. Additional facilities required: None

1003542075

12. Biographical sketches of investigator(s) and other professional personnel (append).

13. Publications. (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

Especially since it is a very real possibility that drugs affecting or having state dependent effects in one procedure will not on the other hand.

C. Training Rats to Discriminate Between Nicotine and Non-Nicotine States

Rats will be trained to discriminate between nicotine and non-nicotine states using a standard two-lever operant chamber. In this schedule animals will be food deprived and initially shaped to press both levers for a food reinforcement. Once training has been completed rats will then be trained to press one lever under the nicotine state and the other lever under the saline state. The schedule of reinforcement will involve a ratio schedule. Most likely, we will be using an FR 5-10. Thus an animal to obtain a food reinforcement, must press the lever 5-10 times for reinforcement. In the initial training procedure, rats will be given nicotine or saline, placed in the operant chamber 5-10 minutes after s.c. injection, and trained on the appropriate lever under each drug state. Experimental sessions will last for 15 minutes; the first two and a half will not be reinforced to determine whether an animal is responding on the correct lever. In this study animals under nicotine will learn to press the correct lever 80-90% of the time which will be our measure of learning. Data is calculated as a percentage of the levers responses on the correct or nicotine lever divided by total responding on both levers. Once training has reached maximum, drugs will be studied as to their ability to generalize to the nicotine state. Learning under the nicotine state is usually defined as rats who will respond on the nicotine correct lever 90% of the time when given nicotine or who respond 30% of the time on the lever when given saline. Thus, a discrimination index or some difference between the two states averages 50-60%.

Once animals have reached training criteria, experimental compounds will be tested on one or two sessions every week. In this situation, drugs

1003542110

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Metabolism of Natural Products of Medicinal Interest	NIH-GM-13246-16	\$ 47154	1.1.73-12.31 73

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Renewal of the above NIH grant	NIH-GM-13246-17 (This will be the terminal year of a 5 year grant period)	\$ 55510	1.1.74 - 12.31.74

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Edward Lecte

Signature *Edward Lecte* Date 7. 25.73

Telephone 612 373 2380
Area Code Number Extension

Checks payable to

University of Minnesota

Mailing address for checks

302 Morrill Hall

University of Minnesota, Minneapolis
55455, Minnesota.

Responsible officer of institution

Typed Name Luther J. Pickrel

Title Assoc. Dean, Graduate School Res.Center

Signature *Luther J. Pickrel* Date
Telephone 612 376 7614
Area Code Number Extension

1003542090

3a

12. Biographical sketch of principal investigator:

Name: Patricia Montague Hudgins

Date of Birth: REDACTED

Place of Birth: REDACTED

Social Security No.: REDACTED

Education: West Virginia University, Ph.D. 1966 (Pharmacology)

Bucknell University

West Virginia University, M.S. 1960

West Virginia University, B.S. 1959 with high honors

Academic Appointments: Associate Professor, Department of Pharmacology
Medical College of Virginia - July 1, 1972Assistant Professor, Department of Pharmacology
Medical College of Virginia - July 1, 1968Instructor, Department of Pharmacology
Medical College of Virginia - February 1, 1966

Professional Society Memberships:

REDACTED

REDACTED

Special Awards: National Institutes of Health traineeship (1963-1965)

13. Publications: (Five most recent and pertinent of investigators; reprints provided).

1. Egle, J.L., Jr., Hudgins, P.M. and Lai, F.M.: Cardiovascular effects of intravenous acetaldehyde and propionaldehyde in the anesthetized rat. *Toxicol. Appl. Pharmacol.* 24:636-644, 1973.
2. Hudgins, P.M. and Stubbins, J.F.: A comparison of the action of acetylcholine and acetylcholine mustard (chloroethylmethylaminoethyl acetate) on muscarinic and nicotinic receptors. *J. Pharmacol. Exp. Ther.* 182:303-311, 1972.
3. Hudgins, P.M. and Putney, J.W., Jr.: Distribution of local anesthetics and the intracellular pH in vascular smooth muscle. *J. Pharmacol. Exp. Ther.* 181:538-546, 1972.
4. Hudgins, P.M., Stubbins, J.F. and Deis, F.H.: Inhibition of norepinephrine uptake and adrenergic antagonism by N-methyl-N-benzylphenylethanolamine and N,N-debenzylphenylethanolamine. *Arch. Int. Pharmacodyn.* 187:236-244, 1970.
5. Hudgins, P.M. and Harris, T.M.: Further studies on the effects of reserpine pretreatment on rabbit aorta: Calcium and histologic changes. *J. Pharmacol. Exp. Ther.* 175:609-618, 1970.

1003542076

TABLE I

Corticosteroid Production in Isolated Adrenal Cortical Cells

	Steroid Production ng/2 hr/2x10 ⁶ Cells
<u>ACTH (pU/ml)</u>	
0	26
12	39
25	60
125	96
<u>Butyryl cyclic AMP (mM)</u>	
0	26
0.1	69
0.25	164
0.5	266
<u>Prostaglandin E₂ (mM)</u>	
0	35
0.1	72
0.25	83
0.5	100

Each set of values was determined from cells obtained from paired adrenals of different cats. Cells were exposed to a given stimulus for 2 hours.

1003542138

16. Other sources of financial support

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Actions of ethanol and acetaldehyde on arterial muscle	Licensed Beverage Industries, Inc.	\$5,900	7/1/72 to 7/1/73

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Acetaldehyde-cardiovascular and cellular actions	National Institutes of Health NSHL 11479	\$85,718	3/1/74 to 2/28/77

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Patricia M. Hudgins

Signature Patricia M. Hudgins Date 6/28/73

Telephone 804-770-4466
Area Code Number Extension

Checks payable to

☒ Raymond Holmes, Jr.

Vice President for Finance

Mailing address for checks

Virginia Commonwealth University

1200 East Broad Street

Richmond, Virginia 23298

Responsible officer of institution

Typed Name Dr. Lauren A. Woods

Title Vice President, VCU/MCV, Health Sciences Div.

Signature L. Woods Date 6/28/73

Telephone 804-770-4001
Area Code Number Extension

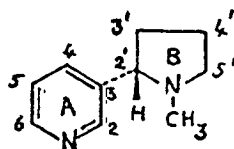
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will be administered, and animals then given a two and a half minute non-reinforced session. These animals will not be given a total 15 min. presentation and will not be reinforced. Under these circumstances drugs having nicotine-like properties will produce an effect in these animals such that animals will press the nicotine correct lever. In this procedure, several doses of each compound can be tested within one month. Doses to be studied in this study will be either equimolar to nicotine, or doses suspected of having CNS effects. CNS effects will also be determined in preliminary studies of each compound, or from data extrapolated from research conducted by other investigators.

Drug antagonism studies can be conducted in the same group of animals. Care will be taken to spread out these studies so that they will not interfere with drug transfer research. It might be useful to use a different population of animals to study either transference or antagonism effects of various drugs. In such studies, dose-response experiments will be conducted in which drugs will be given at a specified time interval before a dosage of nicotine. In general, antagonism studies will be conducted 15 to 30 minutes before a dose of nicotine is administered. Thus, animals will be injected with the suspected compound and then administered nicotine 30 minutes thereafter. Ten minutes after this, the animal will be placed in the operant chamber and given one test session. That is, animals will be given two and one half minutes in the operant chamber, but not given any reinforcement. A drug which will produce an antagonistic effect will block the effects of nicotine such that the animals will respond as if they were given saline, and will press the saline correct lever. In this type of investigation, two types of studies can be conducted in which ¹⁾ a dose response of the antagonist drug can be studied as blocking the effects of nicotine or ²⁾ nicotine can be studied in a dose response fashion in an

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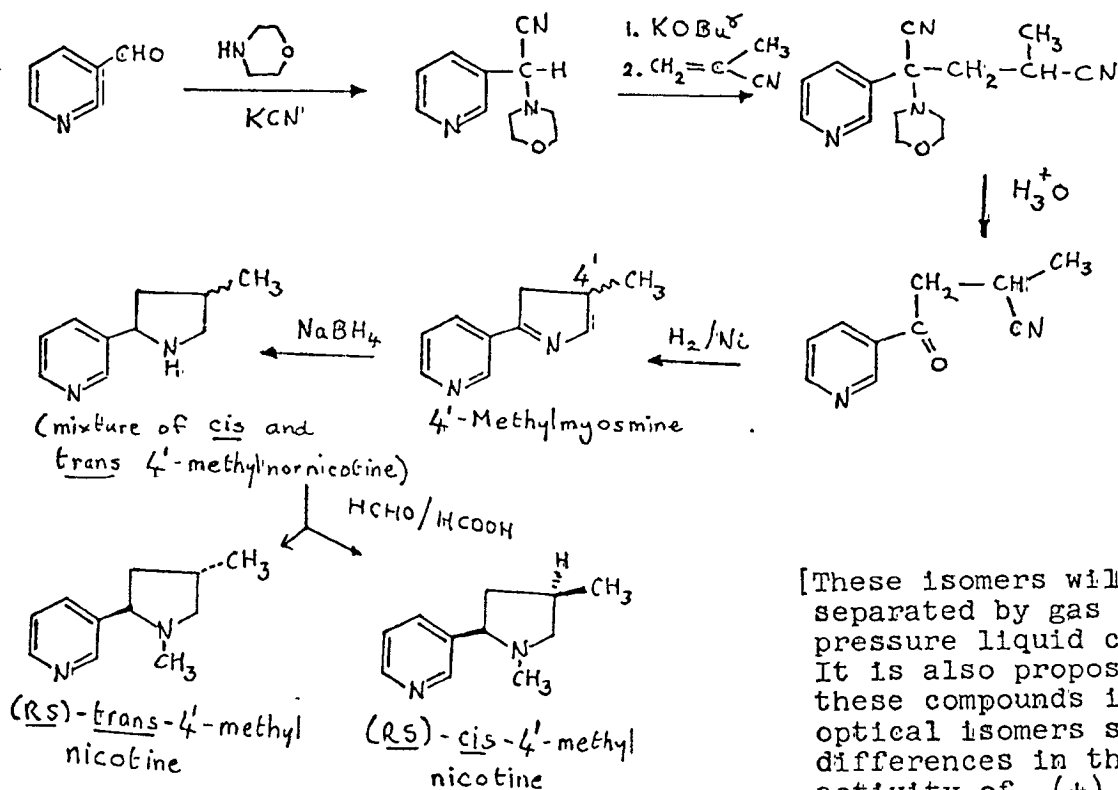
From work already published (cf. F. Haglin, Acta Pharm. Suecica, 4, 117 (1967)) it is clear that certain structural features are required in a molecule if it is to exhibit "Nicotinic Activity" in biological tests. The pyridine ring (A) is essential, however it is possible to substitute this ring at the 5- and 6-positions and retain activity. Substitution at the 2- and 4-positions results in a drastic decrease in activity, and it is suggested that such substitution results in restricted rotation around the C-3~C-2' bond, inhibiting the required interaction of the nicotine molecule with the receptor site (presumably on some membrane) which leads to the biological properties of nicotine. Initially it is proposed to substitute the pyrrolidine ring (B) with methyl groups, having known stereochemistry relative to the pyridine ring. The biological activity of these analogs will be compared with 1-nicotine.



L-Nicotine

9. Details of experimental design and procedures (append extra pages as necessary)

We have recently developed a new synthesis of norm nicotine and myosmine (E. Leete, M. R. Chedekel, and G. B. Bodem, J. Org. Chem., 37, 4465 (1972)) which we feel will be of general use for the synthesis of large amounts of nicotine analogs. The synthetic schemes which we propose to use for the preparation of these analogs are illustrated below.

4'-Methylnicotine

[These isomers will be separated by gas or high pressure liquid chromatography. It is also proposed to separate these compounds into their optical isomers since differences in the biological activity of (+) and (-)

nicotine has been observed (R. B. Barlow and J. T. Hamilton, Brit. J. Pharm. Chem., 25, 206 (1965).]

1003542082

Second

14. First year budget:

A. Salaries (give names or state "to be recruited")

% time

Amount

Leonide Goldstein, D. Sc.

10

Judith M. Nelsen, Ph. D.

100

REDACTED

Technical

Katheleen Pelley, B.A.

100

REDACTED

Sub-Total for A

B. Consumable supplies (by major categories)

Food pellets..... 100.00
 Chart paper..... 100.00
 Behavioral modules..... 200.00
 Chemicals and drugs..... 100.00
 Maintenance of computer..... 200.00

Sub-Total for B

700.00

C. Other expenses (itemize)

Travel to 2 Meetings for P.I. 500.00
 Travel to 1 Meeting for co-P.I. 266.00

Sub-Total for C

766.00

Running Total of A + B + C

REDACTED

D. Permanent equipment (itemize):

None

Sub-Total for D

E. Indirect costs (15% of A+B+C)

E

4,350.00

Total request

REDACTED

15. Estimated future requirements

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2						
Year 3						

1003542056

what other systems these drugs might be effecting. Thus, enzyme inhibitors will be inhibitors will be utilized which will inhibit either brain norepinephrine or brain 5-hydroxytryptamine systems.

BIBLIOGRAPHY

1. Schechter, M. D., and J. A. Rosecrans: CNS effect of nicotine as the discriminative stimulus for the rat in a T-maze. *Life Sciences* 10, 821 (1971).
2. Schechter, M. D., and J. A. Rosecrans: Behavioral evidence for two types of cholinergic receptors in the CNS. *European J. Pharmacol.* 15, 375 (1971).
3. Schechter, M. D., and J. A. Rosecrans: Behavioral tolerance to an effect of nicotine in the rat. *Arch. Int. Pharmacodyn.* 194, 134 (1971).
4. Schechter, M. D., and J. A. Rosecrans: Nicotine as a discriminative stimulus in rats depleted of Norepinephrine or 5-hydroxytryptamine. *Psychopharmacologia* 24, 417 (1972).
5. Schechter, M. D., and J. A. Rosecrans: Effect of mecamylamine on discrimination between nicotine - and arecoline - produced cues. *European J. Pharmacol.* 17, 179 (1972).
6. Schechter, M. D., and J. A. Rosecrans: Lysergic acid diethylamide (LSD) as a discriminative cue: drugs with similar stimulus properties. *Psychopharmacologia* 26, 313 (1972).
7. Schechter, M. D., and J. A. Rosecrans: Nicotine as a discriminative cue in rats: inability of related drugs to produce a nicotine - like cueing effect. *Psychopharmacologia* (In press).

1003542113

TABLE II

Adrenal Cyclic AMP Levels
after Exposure to Nicotine or Acetylcholine

	<u>Conc.</u> <u>M/liter</u>	<u>Exposure</u> <u>Time</u> <u>(min)</u>	<u>Cyclic AMP</u> <u>p/moles/gland</u>		<u>Percent</u> <u>Increase</u>
			<u>Control</u>	<u>Exptl.</u>	
Nicotine	2×10^{-5}	12	250	460	85
	6×10^{-5}	3	425	610	43
	1×10^{-4}	6	763	1600	<u>110</u>
				Mean ± S.E.	79.3 ±19.5
Acetylcholine	6×10^{-6}	5	583	713	33
	6×10^{-6}	3	312	447	43
	2×10^{-4}	10	200	320	60
	2×10^{-4}	8	363	820	<u>126</u>
				Mean ± S.E.	65.5 ±20.9

The increase in cyclic AMP was determined from the value of the stimulated right gland as a percentage of the value of the control left gland.

Each experiment was carried out on a different preparation.

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July 27, 1973

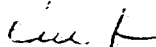
1974-1975 would be helpful -- assuming the Board is willing to take this matter under consideration at the present time.

The present staff remains intact here, except for one of our graduate students who was doing good work, but felt unhappy in the general environment of Richmond. Prior to leaving, he was working with nicotine and its metabolites on aortic strips and intestinal segments. One of our brominated derivatives of nicotine, in his preliminary studies, appeared to have a strong blocking effect on nicotine. We have not repeated the work, but mention it because it bears on the last paragraph of your letter of July 3, 1973, in which interest is expressed in specific and selective antagonists.

If there are any questions in connection with the enclosures, please do let me know.

With all good wishes to you and your colleagues,

Sincerely,



Herbert McKennis, Jr.

acf

P.S. The enclosed renewal application does not bear the signature of an authorized official of the University. As soon as the official signed copy is received I will send it to you.

1003542094

TABLE III

The Effect of Nicotine and Acetylcholine on Cyclic AMP Release
from the Perfused Cat Adrenal Gland

		Cyclic AMP Release pmoles/min		Catecholamine Release µg/min	
		<u>Control</u>	<u>Experi- mental</u>	<u>Control</u>	<u>Experi- mental</u>
1	Acetylcholine $2 \times 10^{-4}M$ for 10 min	1.5	44.4	< .050	22.7
2	Nicotine $6 \times 10^{-5}M$ for 12 min	2.8	6.7	< .050	5.8
3	Nicotine $6 \times 10^{-5}M$ for 5 min	2.8	6.4	-	-
4	Acetylcholine $2 \times 10^{-5}M$ for 7 min	1.2	2.4	-	-

Each experiment was carried out on a different preparation.

The control values were generally obtained from 10-min collection periods.

1003542140

Curriculum VitaeLeonide Goldstein, D.Sc.

Born:

REDACTED

Marital Status:

REDACTED

1921-35 Student at the Conservatoire National de Musique, Paris
1935 Graduated in violin, harmony and composition
1935-36 Military duty, French Army
1936-37 Technical Assistant Institut de Biologie, Paris
1937-39 Research Assistant
1939-40 Non-commissioned Officer, French Army
1940 Undergraduate studies, University of Paris
1941-42 Research Associate Laboratoire de Physiologie School of
Medicine, University of Montpellier, France
1942 Member of the Research Division of the Free French Forces
1942-45 Special assistant to Dr. H.J. Muller, Amherst College,
Amherst, Mass.
1944 B.A. and M.A. Amherst College
1945-47 Assistant Professor University of Paris (Physiology and
Genetics)
1947-53 Acting Director Laboratoire de Biometrie of the French
National Research Council
1951 Doctor of Sciences degree, University of Paris, Sorbonne
1953-58 Assistant Professor, Neurophysiology, Ecole Pratique des
Hautes Etudes, Sorbonne
1958-61 Associate Professor, Pharmacology, Emory Univ., Atlanta, Ga.
1961-64 Neuropharmacologist, Bureau of Research, Neuropharmacology
Section, N.J. Neuropsychiatric Institute, Princeton, N.J.
1964-72 Research Scientist Grade 1, Bureau of Research, Neuropharma-
cology Section, N.J. Neuropsychiatric Institute, Princeton, N.J.
1969-73 Visiting Senior Fellow - Department of Biology - Princeton Univ.
1972 Associate Professor of Psychiatry, Rutgers Medical School,
Piscataway, N.J.
1973 Member Graduate Faculty, Rutgers University, New Brunswick, N.J.
1973 Member Psychobiology Area Graduate Program in Psychology, Rutgers
University, New Brunswick, N.J.

Membership in Scientific Societies:

REDACTED

REDACTED

REDACTED

Honors:

Croix de Guerre (1939-40): Medal of the Free French Forces: Palmes
Academiques (1950). Associate Editor "Research Communications in
Chemical Pathology and Pharmacology."

Listings:

American Men at Science - Who is Who in the East.

Publications:

Author or co-author of 150 papers and abstracts.

5-23-73

1003542066

3b.

Item 12 (continued)

Biographical sketches of the professional personnel

Edward Leete, Principal investigator**REDACTED**

. Obtained a State Scholarship and attended the University of Leeds , obtaining a 1st class honours B.Sc. degree in Colour Chemistry and Dyeing in 1948 . Carried out graduate work in the department of colour chemistry with Professor William Bradley obtaining a Ph.D. degree in 1950 . Thesis title : Mechanism of the formation of indanthrone from 2-aminoanthraquinone. Awarded a travelling scholarship of the Goldsmiths Company and spent two years at the National Research Council of Canada, Ottawa, working with Dr. Leo Marion on alkaloids. In 1952 awarded an NRC postdoctoral fellowship and continued an additional two years with Leo Marion. In 1954 accepted a faculty position at the University of California, Los Angeles in the department of chemistry, instructor 1954-56 , assistant professor 1956-58 . In 1958 accepted a position at the University of Minnesota, assistant professor 1958-60. associate professor 1960-63, professor 1963-

REDACTED**REDACTED**

. 1962-65 - member of the medicinal chemistry study section of the NIH. 1962-65 Alfred P. Sloan Foundation fellow. 1965-Guggenheim fellow - held at the University of Oxford, England. 1965-award D.Sc. degree from the University of Leeds. Notable invited lectureships : NSF lecturer at the University of Arizona, October 1967; Foster lecturer at the University of Buffalo 1969; Symposium lecturer at the 1st Philip Morris Science Symposium 1973 . Author of 131 scientific publications.

George B. Boden, graduate student**REDACTED**

A resident of Minnesota where he went to Blake school. Obtained a B.S. degree from the University of Minnesota in 1962 . He has been a graduate student in my research group for several years and obtained an M.S. degree in 1972. Thesis title : Aberrant metabolism in higher plants : Formation of 5-fluoronicotine from 5-fluoronicotinic acid in *Nicotiana tabacum*. He is currently a Ph.D. candidate. He is an active and enthusiastic worker and has published 4 papers with the principal investigator.

REDACTEDPhilip M. Hoekstra, graduate student**REDACTED**

Obtained a B.A. degree from Dordt College, Sioux Center, Iowa in 1971 . He is currently a 2 nd year graduate student and he is a Ph.D. candidate having passed all the written preliminary examinations . He is currently studying the biomimetic synthesis of nicotine analogs .

REDACTED

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In this approach rats trained under nicotine will enter the shock chamber faster when tested in the non-drug state (group A) than when tested in the drug state (Group B). On the other hand, rats trained under saline will retain the passive avoidance response regardless of drug treatment of the test state. (Groups C & D)

This response has been studied with nicotine and has been observed to produce a classical dissociation of learning or state dependent effect. Under such a test, any drug having a power C.N.S. effect will produce this type of behavior. It is possible that some drugs will produce an symmetrical association, that is, the drug effect would block avoidance in both groups A and C. This would suggest that the drug may also be affecting memory, which would also indicate a powerful CNS effect, and would suggest that such a compound should be studied in greater detail. Of special importance is the fact that this technique will detect C.N.S. drug effects at dosage levels lower than that usually observed in conditioned avoidance behavior, or spontaneous activity experiments.

B. Active Avoidance Studies in Rats and Mice

Another approach to be used in this study, will involve the same general model as described above. In this situation, a rat will be trained to avoid a shock (one way or two shuttle box) in the drug state and performance studied in drug and non-drug states. The design will be similar to the 2 x 2 design described above in the passive avoidance test. The data obtained should be similar, that is if a drug has a strong state dependent effect, then performance of the task learned in the drug state will be lower when tested in the non-drug state. Both avoidance (passive and active) procedures will provide similar types of information. However, at this time it is felt that both procedures will be extremely important in attempting to obtain a complete picture of each drug studied.

1003542109

CURRICULUM VITAE

John Adam Rosecrans

Personal

REDACTED

Education

Primary: Public School Systems of Jamaica, Brooklyn, and Long Beach, N.Y.
Secondary: Long Beach Junior and Senior High School, N.Y.
College: B.S. in Pharmacy, St. John's University of New York.
Graduate School: M.S. (Pharmacology), 1960, University of Rhode Island,
Kingston, Rhode Island
Ph.D. (Pharmacology), 1963, University of Rhode Island,
Kingston, Rhode Island.

Professional Experience

Instructor in Pharmacology (September 1960 - June 1961), College of Pharmacy, University of Rhode Island, Kingston, Rhode Island.

Research Assistant Professor (August 1964 - November 1965), School of Pharmacy, University of Pittsburgh, Pittsburgh, Pennsylvania.

Part-time Instructor in Chemistry (September 1966 - July 1967), New Haven College, New Haven, Connecticut.

Assistant Professor (July 1967 - July 1970), Department of Pharmacology, Medical College of Virginia, Richmond, Virginia.

Associate Professor (July 1970 - Present), Department of Pharmacology, Medical College of Virginia, Richmond, Virginia.

Professional Societies

REDACTED

REDACTED

Awards and Fellowships

The Phi Sigma and Sigma Xi awards at the University of Rhode Island for graduate work leading to the M.S. degree in Pharmacology.

U.S.P.H.S. Predoctoral Research Fellowship (N.I.M.H.), University of Rhode Island (June 1961 - February 1963), Sponsor - Dr. John J. DeFeo.

U.S.P.H.S. Postdoctoral Research Fellowship (N.I.M.H.), University of Michigan (February 1963 - August 1964), Sponsor - Dr. Edward F. Domino.

1003542117

TABLE IV

The Effect of Nicotine on Corticosteroid Production
in Isolated Adrenal Cortical Cells

	<u>Steroid Production</u> <u>ng/2 hr/2x10⁵ cells</u>	<u>Percent</u> <u>Increase</u>
Control	15.4 ± 2.4	-
Nicotine (6 x 10 ⁻⁶ M)	19.7 ± 4.6	28
Nicotine (6 x 10 ⁻⁵ M)	18.7 ± 2.0	21
Nicotine (6 x 10 ⁻⁴ M)	19.9 ± 3.1	29
ACTH (25 µU/ml)	21.9 ± 2.7	42
ACTH + Nicotine (6 x 10 ⁻⁶ M)	21.7 ± 3.5	41
ACTH + Nicotine (6 x 10 ⁻⁵ M)	25.7 ± 2.5	67
ACTH + Nicotine (6 x 10 ⁻⁴ M)	27.7 ± 3.9	80

Each mean value (± standard error) was obtained from paired adrenals of 5 different cats.

Each individual sample was assayed in duplicate or triplicate.

1003542141

Biographical sketches of all principal and professional personnel:

A. STANLEY WELTMAN

Born:

REDACTED

1. Education

Brooklyn College, Brooklyn, New York	B.A.	1941	Biology and Chemistry
Columbia University, New York, New York	M.A.	1949	Zoology
University of Missouri, Columbia, Mo.	Ph.D.	1956	Zoology

2. Experience

<u>Institution</u>	<u>Nature</u>	<u>Year</u>
Laboratories for Therapeutic Research, Brooklyn College of Pharmacy	Endocrinological, Physiological & Pharmacological Research	1956 to present
University of Missouri	Graduate Research Assistant (Zoology, Histology, Genetics)	1952-1956
U.S. Army	Medical & Surgical Technician (Anesthetist)	1943-1946
Beltsville Research Center	Endocrine Studies	1942-1943
Fort Totten Hospital, N.Y.	Laboratory Analyses (Hematology, Urine Analyses and Blood Chemistry)	1941

3. Background

Dr. Weltman is a staff member of the Laboratories for Therapeutic Research and Associate Professor of Pharmacology and Research at the Brooklyn College of Pharmacy, Long Island University, Brooklyn, New York 11216, and an Associate Professor of the Graduate Faculties of Long Island University, Brooklyn Center, Zeckendorf Campus, Brooklyn, New York 11201.

Dr. Weltman had been involved in investigations at the Beltsville Research Center, Beltsville, Maryland, of hormone assays of gonadatropins, estrogens, pituitary extracts, etc. The various studies at times involved hypophysectomies, gonadectomies and adrenalectomies of laboratory animals.

Academically, he is presently engaged in physiological, endocrinological, pharmacological and biochemical research. In addition to research he lectures in physiology, zoology and pharmacology and acts as a sponsor for students involved in graduate research programs in Biology. During the years of academic learning, research and teaching at the various institutions as well as experiences in Army Hospitals and Beltsville Research Center, Dept. of Agriculture, he has become knowledgeable in the areas of zoology, physiology, genetics, biochemistry, etc. He has instructed the biologists and staff in the techniques used to measure and calculate locomotor activity, O₂ consumption, audiogenic-seizure susceptibility, white blood cell counts, estrus cycle, autopsy procedures, as well as other techniques to be used in this study. All staff members realize the strict requirements needed in the care and maintenance of animals for proper scientific research. He has instructed and worked with the biochemist in verifying the validity and applicability of the

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8. Any additional facilities now required? Describe briefly:

No.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

Dr. S. Jaanus, former co-investigator, is no longer connected with the project.

10. Append outline of experimental protocol for ensuing year.

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

Due to the fact that this project began on January 1, 1973, there has not been sufficient time to publish any of the work which has been carried out so far.

1003542129

Table 1: EFFECT OF PROSTAGLANDINS ON THE RELEASE
OF ^3H -NOREPINEPHRINE BY VARIOUS AGENTS
IN THE PERFUSED GUINEA-PIG HEART

Releasing Agent	Control	PGE ₁	PGE ₂	PGF ₂ α
	Δ in ^3H -Norepinephrine dpm/min. \pm S.E.M.			
Nicotine (100 μg)	16,000 \pm 1,500	10,500* \pm 1,121	7,200** \pm 1,500	5,110** \pm 995
KCl (.3M)	49,071 \pm 6,000	37,025* \pm 4,953	32,489** \pm 3,211	36,985* \pm 2,437
Tyramine (300 μg)	11,599 1,200	9,092 1,560	16,264* \pm 102	18,516** \pm 820
Aminophylline (50 mg)	40,023 \pm 5,498	61,360** 2,195	62,514* 10,000	51,178* 5,027

*P < .01

**P < .001

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last several years. However, if no mechanism exists for limiting funds to drug discrimination experiments, then I cannot strongly support funding of this application.

Regarding the budget, I will suggest changes which will constitute an appropriately balanced request for drug discrimination experiments if it is decided to fund such experiments. Salaries should be left as is, consumable supplies should be reduced to 200 rats at \$300 with no mice. Other expenses should stay as is. Permanent equipment should be reduced to \$4,000 which will allow the purchase of two or three operant chambers with required programming and recording equipment. I might also mention that there is apparently an error in the computation of other expenses for years 2 and 3; apparently travel money was deleted from the request for these 2 years. With such revisions, the first year's budget would total \$13,775 and the 3 year total would be \$34,843.

Please let me know if you desire clarification of any of the points above, or if I can be of further assistance in any other way.

Sincerely,



Donald A. Overton, Ph. D.,

DAO/dc

1003542125

PHARMACOLOGY

26
7/30 #929
gh

Comm.

Dr. Gardner
Dr. Jacobson
Dr. Sommers

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., Inc.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8385

Application for Research Grant

(Use extra pages as needed)

JUL 30 1973

7.25.73

1. Principal Investigator (give title and degrees):

Edward Leete, Professor of Chemistry, B.Sc., Ph.D., D.Sc.

2. Institution & address:

University of Minnesota,
Minneapolis
Minnesota, 55455.

3. Department(s) where research will be done or collaboration provided:

Department of Chemistry

4. Short title of study:

Synthesis and Biological Activity of Nicotine Analogs

5. Proposed starting date: 1.1.74

6. Estimated time to complete: 3 years

7. Brief description of specific research aims:

Analogs of nicotine and related tobacco alkaloids will be synthesized for pharmacological testings. Substituted nicotine derivatives will be prepared with known stereochemistry in an effort to determine the relationship between biological activity and the conformation of the nicotine molecule. Biological testing will be carried out by Professor U. S. von Euler and his associates at the Karolinska Institute, Stockholm.

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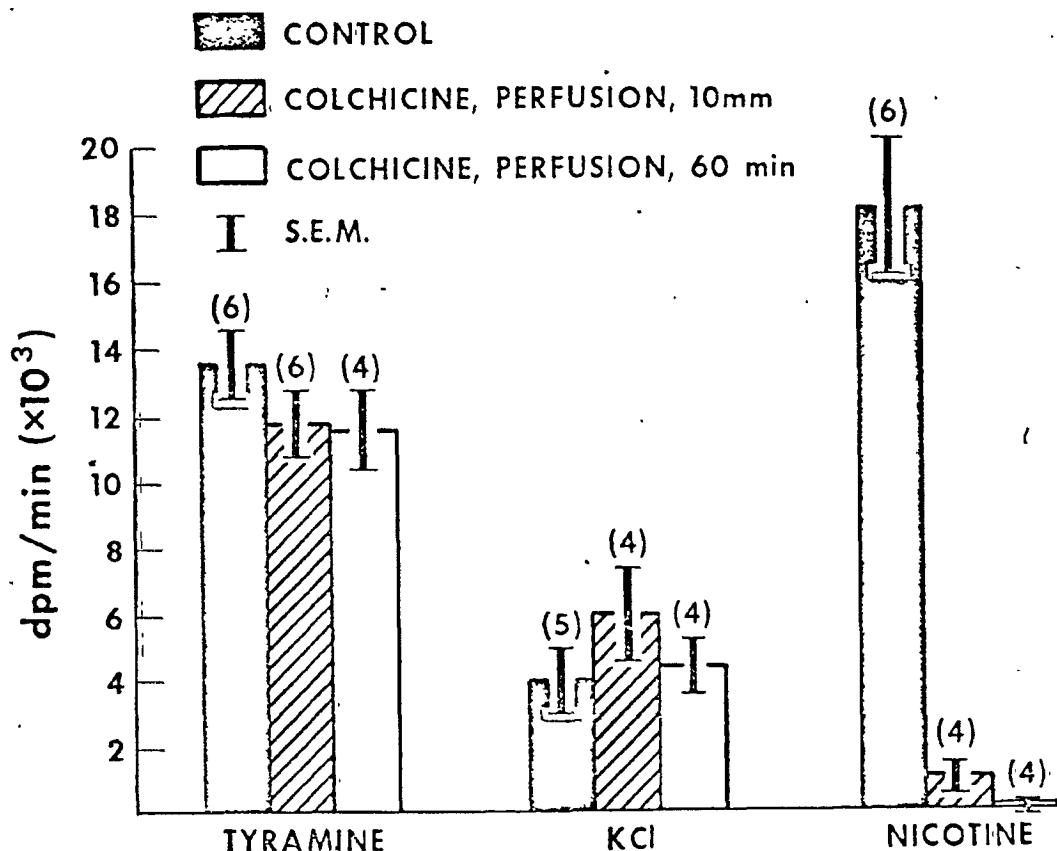


Fig. 2 Shows the effect of tyramine, KCl and nicotine on the release of ^3H -norepinephrine from the perfused guinea-pig heart alone or in the presence of colchicine. Colchicine was perfused for 10 min ($5 \times 10^{-5}\text{M}$) for 60 min ($5 \times 10^{-5}\text{M}$) prior to injecting tyramine, KCl or nicotine. Data is plotted as peak release in dpm/min $\times 10^{-3} \pm$ S.E.M. (I). Numbers above the bars represents number of experiments. It can be seen that colchicine perfused for either 10 or 60 min did not alter the release of ^3H -NE by tyramine or KCl but significantly inhibited the release produced by nicotine.

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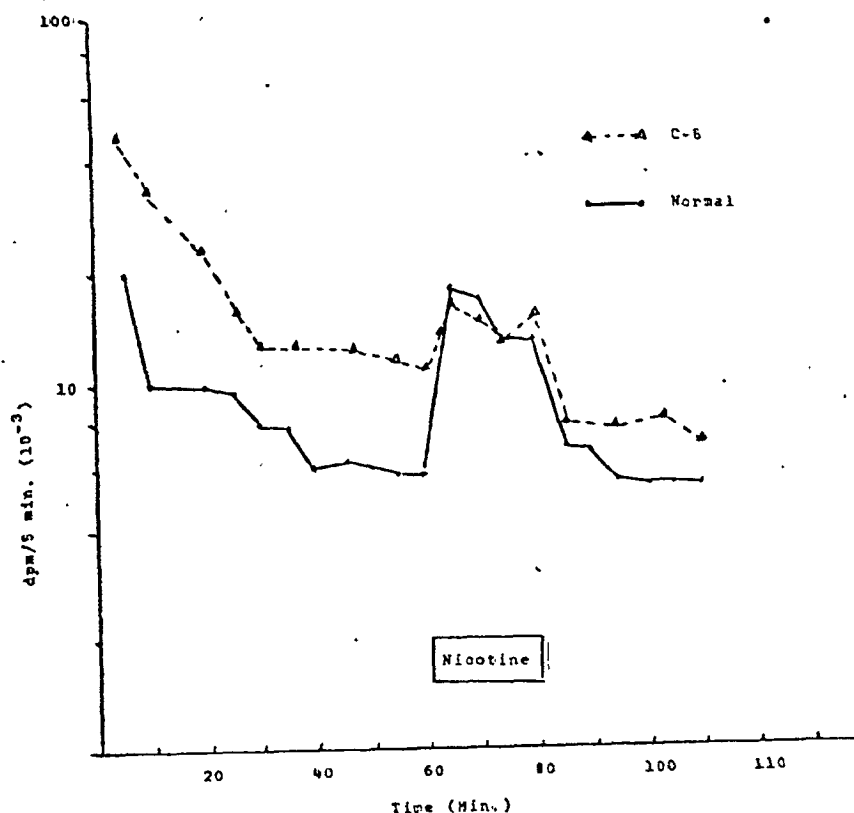


Fig. 6 Depicts the efflux of ^3H -NE from the superfused hypothalamus of the rat. The tissue was prepared in a similar fashion as in Fig. 4 and 5. The solid curve shows the effect of nicotine alone while the dotted curve shows the effect of nicotine in the presence of the ganglion blocking agent, hexamethonium (C-6). It can be seen that the presence of hexamethonium markedly reduces the release of ^3H -NE produced by nicotine.

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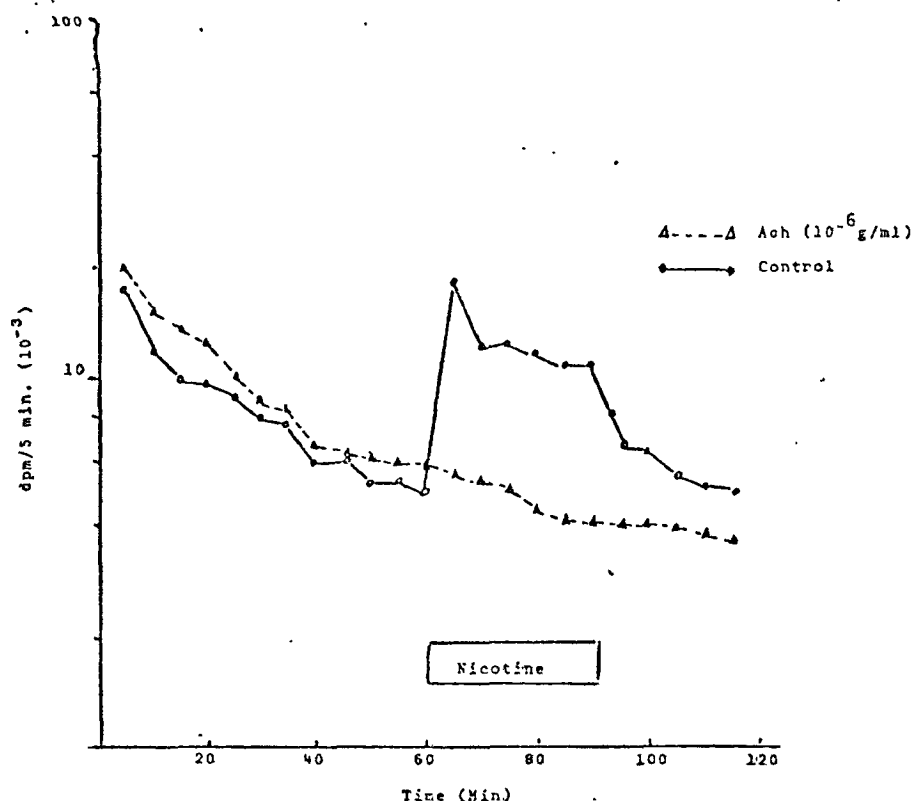


Fig. 7 Depicts the efflux of $^3\text{H-NE}$ from the superfused hypothalamus of the rat. The tissue was prepared in a similar fashion as in fig. 4, 5 and 6. The solid curve shows the effect of nicotine alone while the dotted curve shows the effect of nicotine in the presence of acetylcholine (10^{-6}g/ml). It can be seen that in the presence of this conc. of acetylcholine the effect of nicotine in releasing NE is blocked.

1003542156

8. Brief statement of working hypothesis:

Research conducted in this laboratory (1-8) has shown that learning a specific task can be made contingent upon the drug state an animal is in. Thus, in such situations a rat must be able to detect the drug state it is in in order to obtain a positive or negative reinforcement in a choice situation. The drug, in this situation is acting as a discriminative cue. Another application of this approach involves studying learning in the drug state. This specific approach involves studying how animals learn various tasks in the drug state and then testing their performance rate in the non-drug state. Generally, drugs with powerful behavioral effects produce what is called dissociative learning, i.e. the behavioral performance of a task learned in the drug state will be less than when tested in the non-drug state. Both approaches provide us with the same general information and are considered to be a function of state dependent behavior.

What has been done in this laboratory has been to use this STD paradigm to determine how nicotine is producing its behavioral effects. From this approach, we have found the following: a) the state dependent effect is the result of central cholinergic stimulation, b) nicotine is acting on a specific receptor separate from muscarinic sites, c) brain norepinephrine systems appear to be involved and d) the state dependent effect appears to follow the classical pharmacological mechanisms which depend upon the drug levels at some central site. Essentially, the task we used asked the animal whether it perceived a drug response. In other words, the animal, by the responses it made indicated whether it had perceived nicotine or not. Thus, we have been able to study the drug effect by challenging these responses through various other agents

1003542105

7. Give a Brief Statement of your Working Hypothesis:

A) That measurements of the effect of nicotine on neuronal function can best be determined by comparing the effect on tissues not previously exposed to nicotine with those that have been exposed to nicotine for varying lengths of time. Measurements made on the latter tissue will more closely mimic or correlate with what might be expected in chronic smokers.

B) A second hypothesis is that there may be marked differences in the behavior of neuronal tissue to nicotine when these tissues are taken from animals that have been chronically exposed to nicotine.

8. Details of Experimental Design and Procedures:

A) PRESENT STATE OF KNOWLEDGE IN THE FIELD AND PREVIOUS WORK DONE ON THIS PROJECT.

Nicotine, an important pharmacological ingredient of tobacco, is known to have marked effects on the nervous system. It stimulates autonomic ganglia, the adrenal medulla, the skeletal-neuro muscular junction, certain sensory nerve endings, and has effects in the central nervous system (1-3). In addition, there is also convincing evidence that nicotine produces pharmacological effects by releasing norepinephrine from adrenergic nerve terminals. For instance, this latter effect is seen in preparations devoid of sympathetic ganglia (4,5) and is reduced by procedures which interfere with the functional integrity of the adrenergic nervous system including reserpine (6-9), 6 hydroxydopamine (10), adrenergic blocking agents (4,9,11) and denervation (12).

1003542146

5.

16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project

Source
(give grant numbers)

Amount

Inclusive
Dates

None

PENDING OR PLANNED

Title of Project

Source
(give grant numbers)

Amount

Inclusive
Dates

None

I am thinking about a proposal (nothing prepared yet) to be submitted to the National Cancer Institute to study nicotine agonists and antagonists to alter tobacco smoking behavior.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Edward F. DominoSignature Edward F. Domino Date March 21, 1973Telephone 313 764-9115
Area Code Number Extension

Checks payable to:

The Regents of The University of Michigan

Mailing address for checks

3014 AdministrationUniversity of MichiganAnn Arbor, Michigan 48104

Responsible officer of institution

Typed Name Charles G. OverbergerTitle Vice President for ResearchSignature C. G. Overberger Date 3/27/73Telephone 313 764-1185
Area Code Number Extension

1003542014

16. Other sources of financial support.

List financial support from all sources, including own institution, for this and related research projects

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Effects of a Prostaglandin analogue on brain electrical activity and behavior	Office of Naval Research. Contract # 14-73-C0203	16,000	8/1/73 - 7/31/74
Rutgers Polydrug Treatment Research Project (Consulting Psycho-Pharmacologist)	PHS	134,438	7/1/73 - 7/1/74

PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Microwave radiations: Effects on brain and behavior	PHS RL 01047-01	52,923	1/1/74-12/31/74

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Checks payable to
College of Medicine & Dentistry of N.J.,
Rutgers Medical School
Mailing address for checks
P.O. Box 101
Piscataway, N.J. 08854

Principal investigator

Typed Name Leonide Goldstein, D. Sc.

Signature Leonide Goldstein Date 7/16/73

Telephone 201 832-4416
Area Code Number Extension

Responsible officer of institution

Typed Name Stanley S. Bergen, Jr. M.D.

Title President

Signature Stanley S. Bergen, Jr. Date 7/14/73

Telephone (201) 877-4400
Area Code Number Extension

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3. Specific techniques

A. Passive Avoidance Studies in Mice and Rats

In this procedure an animal is placed in an unfamiliar environment which is bright, and the time to enter a safer darkened area determined. In the case of rats, each is placed in a circular open field (diameter equal to 24 inches) with a bright light suspended 18 inches over the area. Usually a rat will enter into the adjacent dark cage through an opening within 30 seconds. Once the animal has entered the safe area, a guillotine door is closed and the animal presented two one second shocks over a one minute period. Twenty-four hours later, these animals will be returned to the open field and the time for entering the cage shocked in 24 hours previously determined. Generally, rats presented the shock prior, will not enter the cage easily. Because of this, a 300 second maximum exposure time is utilized. The average time or latency for entering is about 220 seconds. With mice the apparatus used is smaller, and the cut off latency on the testing day is 600 seconds. In the experimental design a 2 x 2 drug saline paradigm is utilized. That is, rats are trained and tested under all conditions. (Table presented below).

Group	Trained	Tested
A	Nicotine	Saline
B	Nicotine	Nicotine
C	Saline	Nicotine
D	Saline	Nicotine

1003542108

be reversibly inhibited.

tissue, those models of direct functional significance to be studied are as

(3) 6-Hydroxydopamine can be administered in successive doses of approximately 300 mg to achieve an irreversible denervation of catecholamine containing storage granules in cardiac tissue. Although there has been no previous data concerned with the use of this irreversible inhibitor its direct administration can achieve approximately 85-95% reduction in catecholamine storage by such tissue.

(4) L-DOPA is the precursor of dopamine, which when administered peripherally, will account for increased central dopamine levels. Peripheral administration (200 mg/kg., i.p.) of dopamine will result in increased tissue uptake and conversion to norepinephrine to offer another alternative for increasing cardiac catecholamine levels.

(5) 6-Methoxytetrahydro- β -carboline is a β -carboline derivative, which following parenteral administration (5 mg/kg) will lead to highly significant increases in 5-hydroxytryptamine (5-HT), particularly in platelets, platelet-enriched plasma, and endochromaffin cells of the gastrointestinal tract. As such this compound provides an extremely potent pharmacological tool by which 5-HT, stored in these sites may be increased. Such an increase shows a peak effect by two hours following treatment with several hundred per cent increase being shown in these sites.

(6) 6-Hydroxy 5-Hydroxytryptamine is a recently synthesized substrate which, when administered parenterally, produces a marked and long lasting depletion of 5-HT concentration; the duration of this depletion is as long as 30 days, depending upon the dose utilized and the direct site of investigation. This compound will prove to be extremely useful in evaluating platelet or endochromaffin cell 5-HT depletion.

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1003542145

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885

Application For Renewal of Research Grant

(Use extra pages as needed)

First Renewal ☒

Second Renewal ☐

Date: 7/10/73

1. Principal Investigator (give title and degrees):

Ronald P. Rubin, Ph.D., Associate Professor of Pharmacology

2. Institution & address:

State University of New York
Downstate Medical Center
450 Clarkson Avenue
Brooklyn, New York 11203

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

The Action of Nicotine on the Adrenal Gland

5. Proposed renewal date: 1/1/74

6. How results to date have changed earlier specific research aims:

The finding that nicotine can potentiate the steroidogenic effect of ACTH is of significance in light of the fact that adrenal hormones have marked effects on many physiological and pathological processes, and is of central importance in the homeostatic mechanisms which are activated during prolonged stress. Thus, one of the primary aims of this project will be to carry out investigations to ascertain the extent and the nature of the mechanism by which nicotine enhances the activity of ACTH.

Since nicotine-induced catecholamine release is associated with increased cyclic AMP levels, experiments will also be carried out to ascertain whether the increases in cyclic AMP are directly responsible for the enhanced rate of secretion.

7. How results to date have changed earlier working hypothesis:

The original working hypothesis was to ascertain whether nicotine can affect adrenocortical function. Our preliminary experiments indicate that it does potentiate the activity of ACTH, and therefore our efforts are now being devoted to investigating the nature of this action of nicotine.

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4. Travel

This item of the budget will enable the Principal Investigator to attend one meeting of the Pharmacological Society.

5. Other Expenses

These include a request for publication costs and commuter time.

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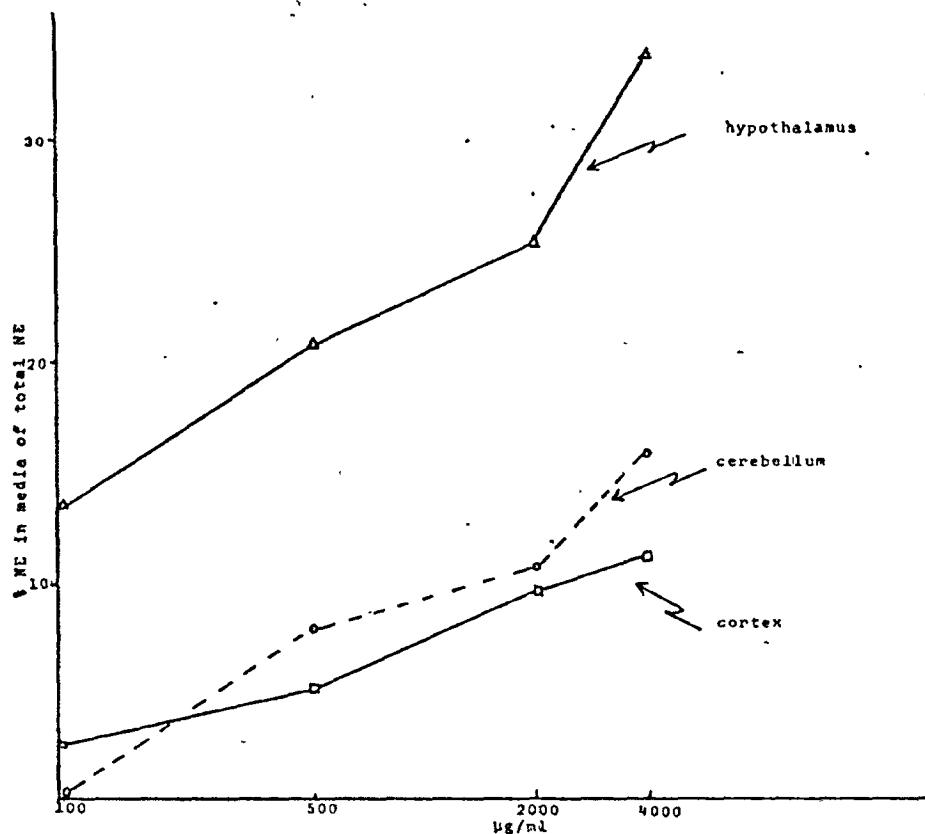


Fig. 3 Depicts dose response curves plotting the effect of nicotine on the release of ^3H -NE from incubated chopped brain slices from 3 different brain regions. Conc. of nicotine in $\mu\text{g/ml}$ is plotted on abscissia and % NE in the media as % of total NE on the ordinate. It can be seen that there is a dose related release of ^3H -NE from all three brain regions with the release being greatest from hypothalamus.

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#927 - WILSON

1003542184

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Ronald P. Rubin (Principal Investigator)

50

Technical

100%

\$11,000.*

* Includes 19% for fringe benefits

Sub-Total for A \$11,000.

B. Consumable supplies (by major categories)

Glassware

500.

Radioactive Chemicals

1,000.

Animals

1,000.

Other Miscellaneous Laboratory Items

500.

Sub-Total for B \$3,000.

C. Other expenses (itemize)

Travel (to attend scientific meetings)

500.

Sub-Total for C \$500.Running Total of A + B + C \$14,500.

D. Permanent equipment (itemize)

Sub-Total for D

E \$2,175.

E. Indirect costs (15% of A+B+C)

Source: <https://www.industrydocuments.ucsf.edu/docs/gymn8160>

1003542130

Awards and Fellowships (continued)

Postdoctoral Trainee in Psychiatry, Yale University (November 1965 - July 1967), Sponsor - Dr. Daniel X. Freedman and Dr. Roger K. McDonald.

Research Interests

Biochemical and Psycho-pharmacology with special interests in correlations between biochemical, electrophysiological and behavioral events associated with CNS acting drugs.

Research Grants

Research conducted by Dr. John A. Rosecrans has been largely supported by the AMA-ERF Committee on Tobacco and Health. Dr. Rosecrans has also had some support from the NIMH and has one grant approved, but not yet funded.

Journal Activities

Dr. Rosecrans is Regional Editor for the journal, Pharmacology, Biochemistry and Behavior.

Educational Activities at the Medical College of Virginia

Professional: involved in teaching CNS Pharmacology at MCV; including Schools of Pharmacy, Medicine, and Dentistry.

Graduate: has presented courses in psychopharmacology involving graduate students in Pharmacology and Psychology. Dr. Rosecrans is also currently involved in a collaborative course on drug dependence with the Department of Sociology. He is also on the graduate committee for several students in Psychology and has two students working toward Ph.D. degrees in Pharmacology.

Undergraduate Education: has been involved in presenting a pharmacology course to undergraduate students at Virginia Commonwealth University during the last three years. Dr. Rosecrans has been coordinator of this program during this period. He has also introduced a new undergraduate course on drug dependence. This latter program has also enabled several sociology and psychology majors to conduct independent projects in pharmacology.

Community Education: Dr. Rosecrans was coordinator of a teacher-training program involving 250 students. This was in conjunction with the Council on Drug Abuse Control in Richmond and was supported by LEAA.

Adult Education: Dr. Rosecrans has been involved with the education of adults via the extension division of Virginia Commonwealth University. Three 10-hour classes have thus far been presented.

Additional Activities: Dr. Rosecrans has been involved in two additional programs. The first involves a medical elective to assist students in learning about drug abuse problems. In the second program, a summer study program was established in which three pharmacy students studied in the area of drug abuse. In this program students work part-time at the Medical

1003542118

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

July 24, 1973

Grant application No. 869R1

TO: The committee comprising Drs. Gardner, Meier and Sommers

SUBJECT: Ronald P. Rubin, Ph.D., SUNY, Downstate Medical Center, Brooklyn
First Renewal Application #869R1
"The Action of Nicotine on the Adrenal Gland"

History

Grant #869, for calendar 1973, was awarded in the amount requested (\$21,300.). Priority in competition for two additional years was recommended.

Application #869R1 requests \$16,675., exactly the amount initially estimated.

Documents Submitted

Attached is application dated 7/10/73, including Progress Report #1, 1/1/73 - 6/31/73 (total 14 pages).

Comment

The application notes that Dr. S. D. Jaanus is no longer associated with the study. Dr. Jaanus, a recent Ph.D. in Pharmacology, is stationed at a different institution in this university complex.

FWN:gh

Enclosure

FWN
F.W.N.

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Investigation of the importance of central biogenic amines on the behavioral effects of nicotine	A.M.A.E.R.F.	\$88,520	2/1/73 - 1/31/76

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Effects of acute and chronic methadone treatment on Psychological and endocrine parameters in animals	N.A.D. MH-22261-01	\$160,959	9/1/72-8/31/75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made"

Checks payable to:

Mr. Walter P. Lossing
Comptroller-Treasurer
Mailing address for checks
Virginia Commonwealth University
Medical College of Virginia
1200 East Broad Street
Richmond, Va. 23298

Principal investigator

Typed Name Dr. John A. Rosecrans
Signature John A. Rosecrans Date 4/30/73
Telephone 703 770-4691 none
Area Code Number Extension

Responsible officer of institution

Typed Name Dr. M. Pinson Neal, Jr.
Title Provost VCU/VCV
Signature M. Pinson Neal, Jr. Date 5/2/73
Telephone 703 770-5150
Area Code Number Extension

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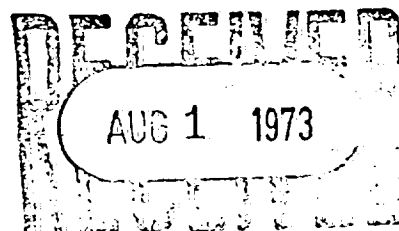


Virginia Commonwealth University

Medical College of Virginia

July 27, 1973

Dr. Robert C. Hockett
Research Director
The Council for Tobacco Research
633 Third Avenue
New York, New York 10017



Dear Bob:

I enclose a renewal application for grant no. 869, which became effective October 1, 1972 and runs through September 30, 1973. Also enclosed is a progress report (30 copies), meeting abstracts, and manuscripts in press or partially completed.

In bringing all of this material together I have tried to be fully responsive to your letter of July 3, 1973, which points out specific targets deemed important by the Council. I hope that you will find, as indicated in my letter of July 10, 1973 that we have been hitting at these targets throughout the period of the grant. And, we hope to continue with vigor.

You noted in your letter the rather general title of the grant. I have not changed the title, but will be glad to do so. I think the word *allied*, which appears in the title, may be restrictive, although perhaps not completely definitive.

In your letter you raised the important "key" question about the extent to which metabolites of nicotine may be responsible for physiological and pharmacological effects that have been attributed heretofore to nicotine itself. This has been discussed at various points in the renewal application and the report. I hope that there has been a satisfactory handling of this most important topic.

Mr. Hoyt's letter of October 18, 1972 stated that the Board had recommended a renewal of the grant for the year past September 30, 1973 (with prior consideration in competition for available funds) at the level of \$60,000. In completing the application for renewal, I followed the original proposal figure of \$74,184 for the second year. This has been done with the hope that additional funding may be possible.

Another consideration is that there is a hope that the present well-trained staff will remain and not try to find other work towards the end of 1973-1974, feeling that funds will be no longer available. Perhaps some indication, if this request is in order, of prior consideration for

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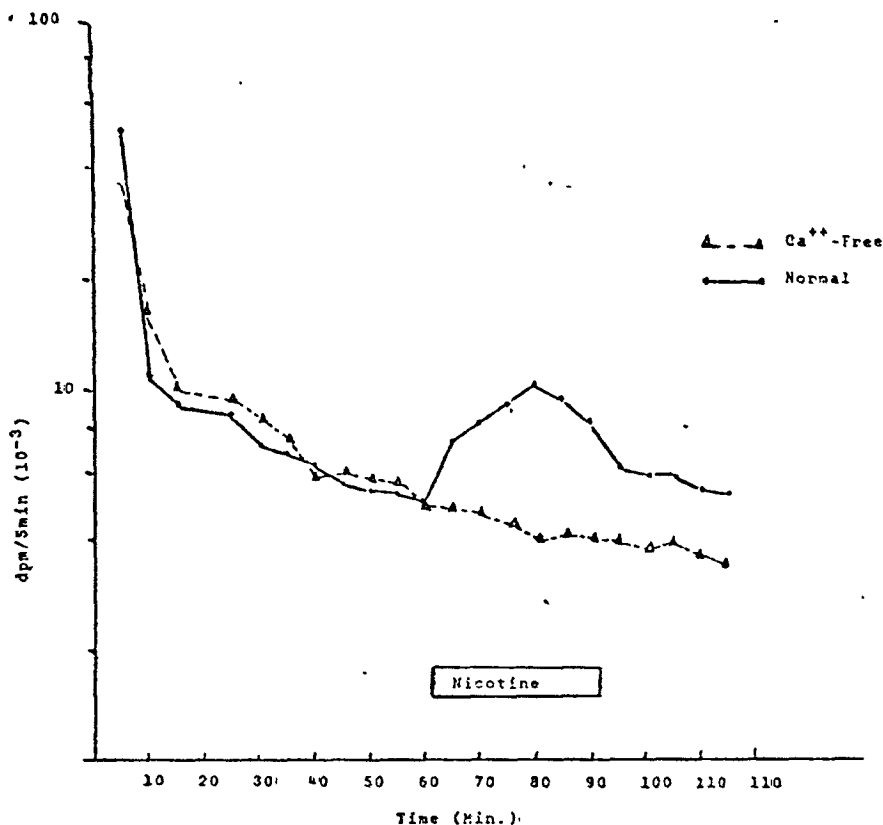


Fig. 5 Depicts the efflux of ^3H -NE from the superfused hypothalamus of the rat. The hypothalamus was dissected out and prepared for superfusion according to the procedure described for the medulla-pons in Fig. 4. Data is plotted in a similar fashion as dpm/5 min (10^{-3}) vs time in min. The solid curve shows the effect of nicotine in slices perfused with normal medium, the dotted curve depicts slices perfused with a solution devoid of Ca^{++} . It can be seen that nicotine produces an increase in the release of ^3H -NE. Removal of Ca^{++} from the perfusion solution completely blocks the nicotine induced release of ^3H -NE.

1003542154

May 31, 1973

Grant Application No. 467C

To: The committee comprising Drs. Bing, Gardner and Jacobson

Subject: Thomas C. Westfall, Ph.D., University of Virginia, Charlottesville
Continuation application No. 467C
"Action of Nicotine on Peripheral and Central Neurons in Animals
Chronically Exposed to Nicotine"

History

This investigator has been supported by CTR since 1965 through Grant No. 467 with renewals, continuations and supplements. The most recent grant ended March 31, 1973.

Application No. 467C requests \$21,953 plus one additional year.

Documents Submitted (attached)

1. Application dated February 1, 1973.
2. Reprints of publications #16, #19, and #22 listed under item 12, page 29 of the application.

Comment

Attached are copies of evaluations provided by Walter B. Essman, M.D., Ph.D. and by Larissa A. Pohorecky, Ph.D.

F.W.N.

FWN:wg
Encls.

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14. First year budget

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s), even if no salary requested)

% time

Amount

Principal Investigator (Patricia Hudgins)

20

REDACTED

Technical

Lab Specialist B (Jacqueline Beckner)

100

Dish Washer - Lab Aide (to be recruited)

60

REDACTED

Sub-Total for A

B. Consumable supplies (by major categories)

Animals (rats and rabbits) - \$2,500

Chemicals and Drugs - \$ 500

Isotopes and related supplies \$1,000

Glassware and hardware - \$ 500

Sub-Total for B

\$ 4,500

C Other expenses (itemize)

Animal care and feeding \$ 300

Equipment Service and maintenance \$ 300

Publication costs (art work and duplication) \$ 250

Laundry \$ 70

Travel to scientific meetings \$ 250 (one per year)

Sub-Total for C

\$ 1,170

REDACTED

Running Total of A + B + C

D Permanent equipment (itemize)

Sub Total for D

E

\$ 2,250

REDACTED

E Indirect costs (15% of A+B+C).

Total request

15 Estimated future requirements

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2	REDACTED	\$4,725	\$1,529		\$3,189	REDACTED
Year 3		\$1,961	\$1,395		\$3,348	

1003542077

25. Brase, D. A. and Westfall, T. C. Stimulation of phenylalanine hydroxylase activity by short chain alcohols. *Pharmacologist*, 13: 193, 1971.
26. Westfall, T. C. Studies on the mechanism of nicotinic agents on adrenergic nerve terminals. *Pharmacologist*, 13: 229, 1971.
27. Westfall, T. C. Action of beta-adrenergic receptor blocking agents on the turnover of norepinephrine in heart and brain. *Fed. Proc.* 31: 567, 1972.
28. Atuk, N. O. and Westfall, T. C. Reduced catechol-o-methyl transferase activity in the liver and increased pressor response to norepinephrine. *Am. Soc. Clin. Invest.* 55, 1972.
29. Westfall, T. C. Further studies on the mechanism of norepinephrine release by nicotine in the perfused guinea-pig heart. *Proceed. Fifth Internat. Congress on Pharmacology*, 1972, San Francisco.

1003542177

The effect of nicotine on ACTH induced steroid production in these isolated cortical cells was investigated. Nicotine produced a small, but consistent increase in steroidogenesis (20-30%), which was not related to its concentration (Table IV). Moreover the alkaloid not only augmented the basal rate of steroidogenesis but also potentiated the steroidogenic response to a submaximal concentration of ACTH (25 μ U/ml) in a dose-related manner (Table IV). Due to time limitations, the potentiating effect of nicotine was tested only on one rather low ACTH concentration; and it is possible that the enhancement by nicotine may be even more striking at ACTH concentrations which produce more marked effects on steroidogenesis. In any event, although there is some evidence in the literature that the rise in plasma corticosteroids produced by nicotine is an indirect effect via the hypothalamic-pituitary pathway (Kershbaum *et al.*, 1968), the experiments which we have conducted up to now indicate that nicotine can exert a direct effect on adrenocortical activity which is apparently independent of extracortical factors.

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14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Significance of Metabolites Pyridylalkylamines	National Institutes of Health	\$3,732	10-1-71 through 8-31-73
Factors Controlling the Development of Pharmacologically Active Derivatives of Nicotine	AMA-ERF	45,750	7-1-73 through 6-30-74

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
No other grant applications are planned or pending at the present time.			

It is understood that the investigator and institutional officers in applying for a grant have read and accepted the Council's "Statement of Policy, Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name Dr. Herbert McKennis, Jr.

Signature [Signature] Date 2-27-73

Telephone 804-770-4406
Area Code Number Extension

Responsible officer of institution

Typed Name M. Pinson Neal, Jr., M.D.

Title Provost
Signature [Signature] Date April 1973

Telephone 804-770-5150
Area Code Number Extension

Checks payable to

Walter Lossing, Comptroller
Medical College of Virginia

Mailing address for check:

1200 East Broad Street
Richmond, Virginia 23298

1003542101

11. Sackler, A.M., Weltman, A.S., Owens, H., Kreger, A.S. and Jacobs, R.: Endocrine Differences of Audiogenic-Seizure Susceptible and Resistant Wistar Rats. *Amer. Zool.* 2:553, 1962.
12. Sackler, A.M., Weltman, A.S. and Owens, H.: Effects of Lysergic Acid Diethylamide on the Total Leukocytes and Eosinophils of the Female Rat. *Nature* 199:1194, 1963.
13. Weltman, A.S. and Sackler, A.M.: Effect of Lysergic Acid Diethylamide (LSD-25) on Growth, Metabolism and the Resistance of Male Rats to Histamine Stress. *J. Pharm. Sci.* 54:1382-1384, 1965.
14. Weltman, A.S. and Sackler, A.M.: Metabolic and Endocrine Effects of Lysergic Acid Diethylamide (LSD-25) on Male Rats. *J. Endocrinology* 34:81-90, 1966.
15. Sackler, A.M. and Weltman, A.S.: Effects of Vibration on the Endocrine System of Male and Female Rats. *Aerospace Med.* 37:158-166, 1966.
16. Sackler, A.M., Weltman, A.S. and Owens, H.: Endocrine and Metabolic Effects of Lysergic Acid Diethylamide On Female Rats. *Toxicology and Applied Pharm.* 9:324-330, 1966.
17. Weltman, A.S., Sackler, A.M. and Sparber, S.B.: Endocrine, Metabolic and Behavioral Aspects of Isolation Stress on Female Albino Mice, *Aerospace Med.* 37:804-810, 1966.
18. Weltman, A.S., and Sackler, A.M.: Timidity and Metabolic Elimination Patterns in Audiogenic-Seizure Susceptible & Resistant Female Rats. *Experientia* 22:627-629, 1966.
19. Weltman, A.S. and Sackler, A.M.: Metabolic and Endocrine Function in Whirler Mice. *Proc. Exp. Biol. & Med.* 123:58-62, 1966.
20. Sackler, A.M. and Weltman, A.S.: Metabolic and Endocrine Differences between the Mutation Whirler and Normal Female Mice. *J. Exp. Zool.* 164:133-140, 1967.
21. Sackler, A.M. and Weltman, A.S.: Effects of Isolation Stress on Peripheral Leucocytes of Female Albino Mice. *Nature* 214:1142-1143, 1967.
22. Sackler, A.M., Weltman, A.S. and Kreger, A.S.: Metabolic and Endocrine Aspects of Audiogenic-Seizure Susceptibility in Female Rats. *Exp. Med. & Surg.* 24:258-269, 1966.
23. Weltman, A.S., Sackler, A.M., Schwartz, R. and Strozman, S.: Effects of Isolation on Maternal Aggressiveness and Body Growth Rates of Offspring. *Experientia* 23:782, 1967.
24. Weltman, A.S., Sackler, A.M. and Owens, H.: Effects of Levels of Audiogenic-Seizure Susceptibility on Endocrine Function of Rats. *Physiology and Behavior* 3:281-284, 1968.
25. Weltman, A.S., Sackler, A.M., Schwartz, R. and Owens, H.: Effects of Isolation Stress on Female Albino Mice. *Laboratory Animal Care* 18:426-435, 1968.
26. Sackler, A.M., Weltman, A.S., Schwartz, R. and Steinglass, P.: Pre-maternal Isolation Effects on Behaviour and Endocrine Function of Offspring. *Acta Endocrinologica* 62:367-384, 1969.

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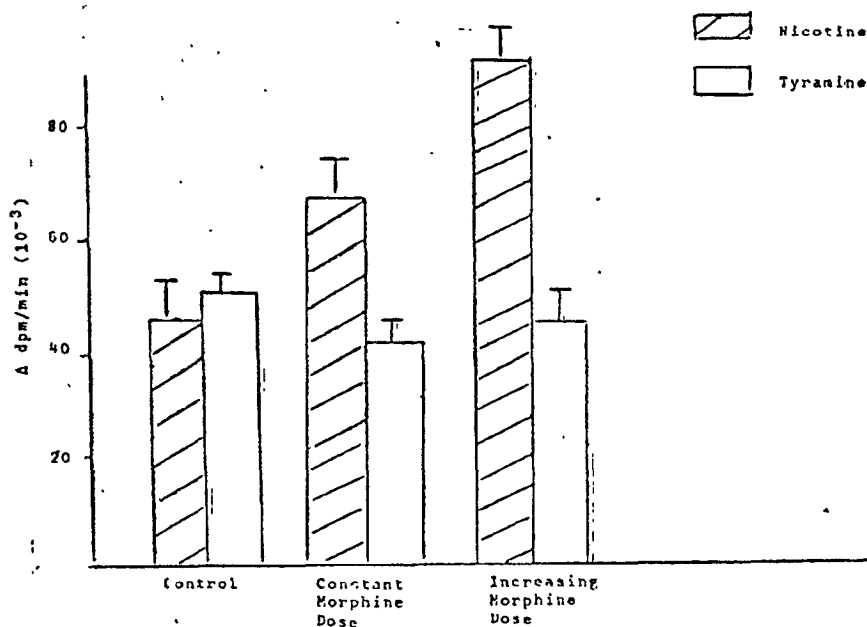


Fig. 8 Depicts the release of ^3H -NE from the perfused rabbit heart by nicotine (100 μg) in hearts obtained from control rabbits, rabbits treated with a constant dose of morphine of 15 mg/kg for 5 weeks or increasing doses of morphine up to 90 mg/kg for 5 weeks. Data is plotted as total amount of NE released in dpm/min (10^{-3}) to a 1 min injection of nicotine or tyramine.

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However, at least half of the proposed work involves using the 2x2 experimental design with active and passive avoidance tasks. I think this is a poor choice for several reasons. To my knowledge, the discriminable effects of nicotine have never been shown to be strong enough to produce obvious state dependent learning in 2x2 experiments, except in Rosecrans own labs. Even in his experiments, he obtains asymmetrical state dependency (below) instead of the symmetrical effect which would be useful in the present research. In previous studies with drugs producing stronger state dependency effects, successful 2x2 parametric studies and comparisons of drugs have not been frequently accomplished due to the various sources of experimental noise and artifact which are intrinsic to this design. Conflicting results have been obtained in various laboratories.

The basic weakness of the 2x2 design results from the fact that a variety of different drug effects influence performance in such experiments. These include drug effects on memorization, on activity level, on performance efficiency, on exploratory behavior, in addition to state dependent learning. It is difficult or impossible to sort out consequences of these various drug effects so as to determine which individual effects were present or to what degree. No complete remedies to the limitations to the 2x2 design are available at present, and in his application, Rosecrans does not indicate that he will use even the partial remedies which are presently available. Hence I believe that the screening experiments described on pages 5-6 of the application will not yield useful results.

An additional problem for the proposed 2x2 experiments is raised by the pilot data which Rosecrans summarizes in paragraph 1 on page 6. These data indicate that nicotine produces an effect which has been called asymmetrical state dependent learning. However, drug discriminations can only be based on symmetrical state dependent learning as the asymmetrical effect allows animals to recall both responses when in the drug condition and hence, does not allow differential responding. Rosecrans proposes to use the 2x2 experiments as a screening device to determine which drugs should be investigated in drug discrimination experiments, and I do not believe they will be useful for this screening. Indeed, I think the 2x2 experiments may be sufficiently misleading so that it would be better to proceed directly to drug discrimination experiments with all compounds of interest to the investigator.

Perhaps I should say that drug discrimination procedures are extremely laborious by comparison with 2x2 experiments, and it is apparently the applicant's intention to speed up his research by initially using the 2x2 design in order to limit the amount of drug discrimination training which must be performed. Obviously I disagree with this decision

Obviously this application places me in some conflict as regards my recommendation. The PI has recently done some extremely important work in this area using drug discrimination procedures and I believe that further work of this type is in the best interest of the Tobacco Research Council. However, I think the PI's recent adoption of the 2x2 design is a serious mistake. If there is mechanism by which the Tobacco Research Council can assure itself that granted monies will be used for drug discrimination studies, then I definitely recommend funding as the PI's recent accomplishments in this area are among the most important reported in the

References alluded to in the Protocol for the Ensuing Year and in the Summary Progress Report.

- Carchman, R.A., Jaanus, S.D. and Rubin, R.P. *Molec. Pharmacol.* 7, 491 (1971).
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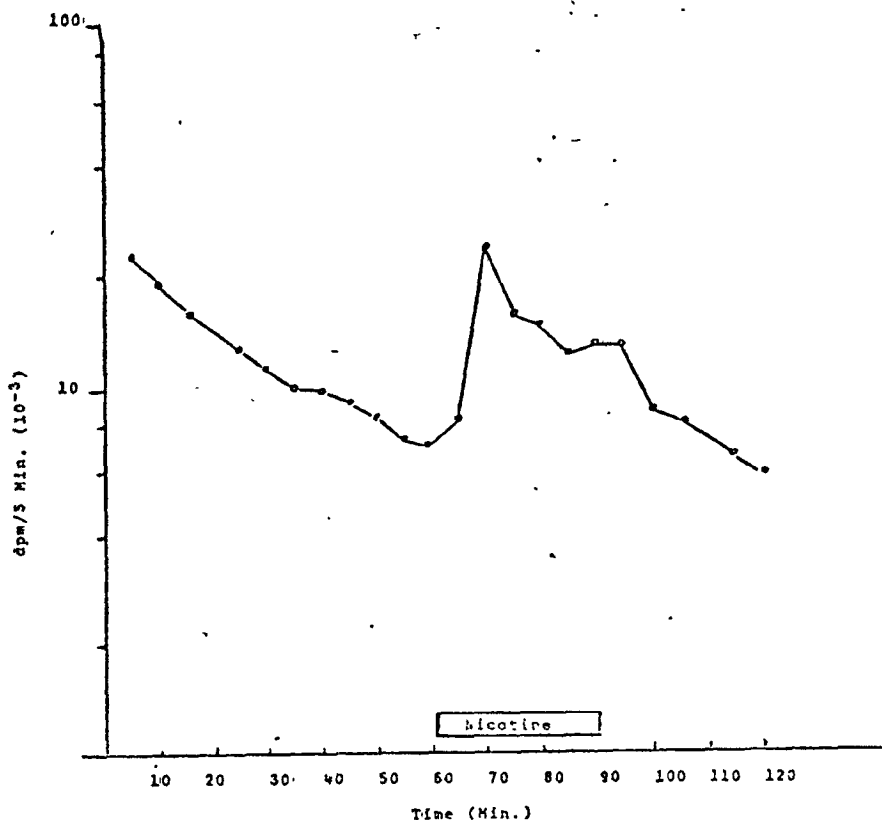


Fig. 4 Depicts the efflux of ^3H -NE from the superfused medulla-pons of the rat. The medulla-pons was chopped into .3mm slices in two directions, incubated with ^3H -NE, washed and layered on Whatman No. 1 filter paper, placed in a Millipore Filter holder. The chopped slices were then superfused with Krebs-Henseleit solution at a constant flow of 0.6 ml/min and the perfusate continuously collected and analyzed for ^3H -NE. Data is plotted as dpm/ 5 min (10^{-3}) against time in min. Following 60 min. Nicotine in a conc. of 1mM was added to the perfusion solution for 30 min. It can be seen that the addition of Nicotine produced a marked increase in the release of ^3H -NE.

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and ^{14}C -tryptamine (New England Nuclear, (S.C. 10 mc/mmole) and the ^{14}C -indoleacetic acid formed extracted into toluene and counted by scintillation spectrometry. Blanks will be prepared by placing them in a boiling water bath for 3 min.

Catechol-o-Methyl Transferase Activity

COMT activity will be measured according to the method of Krakoff et al. (31). One g of tissue (heart or liver) will be homogenized in 4 ml of 1.15% KCl and centrifuged for 10 min. at 10,000 g. An aliquot of the supernatant fraction will then be added to an incubation mixture containing 0.5 M phosphate buffer, 2 M MgCl_2 , 50 μg epinephrine and 1 μg of 5-adenosyl-L-methionine methyl ^3H (New England Nuclear).

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volume will be 2.0 ml. At the end of the incubation, the samples will be centrifuged as described above, the supernatant is discarded and the pellet resuspended in 3.0 ml of the physiological salt solution by mixing in the centrifuge with a Vortex-Genie mixer. This procedure will be repeated 3 times. The fourth suspension is incubated at 37°C for 20 min. After centrifugation and removal of the medium the tissue is layered on Whatman No. 1 filter paper and placed in a Millipore filter holder jacketed with warm water to maintain temperature. The chopped tissue is then superfused at a constant flow of 0.6 ml/min. by means of a Harvard perfusion pump. The perfusate effluents are collected at 5 min. intervals, separated by alumina column chromatography as described earlier and ^3H -norepinephrine, ^3H -dopamine, or ^3H -serotonin counted by liquid scintillation spectrometer. The tissue will be perfused for approximately 1 hour until the perfusate effluent is very constant, the tissue is then switched to a medium containing nicotine in the presence or absence of various drugs and the perfusate effluent continuously collected and counted.

It has been demonstrated that brain tissue is quite viable following such a procedure and can be used as a valid method for measuring the release of transmitters from neural tissue (26). We have demonstrated that nicotine will release ^3H -norepinephrine from various brain regions including hypothalamus, cortex, cerebellum, and medulla-pons using such a technique (Figs. 3-7). The effect of nicotine on the release of dopamine or serotonin is unknown but will be investigated in the proposed study.

1003542163

ENDOGENOUS NOREPINEPHRINE CONTENT
OF VARIOUS BRAIN REGIONS

Brain Region	Norepinephrine Content $\mu\text{g/g} \pm \text{S.E.M.}$
Medulla - Pons	$0.80 \pm .21$
Cerebellum	$0.32 \pm .08$
Cortex	$0.29 \pm .09$
Midbrain	$0.54 \pm .09$
Striatum	$0.53 \pm .12$
Hypothalamus	$1.63 \pm .19$

Fig. 10

REPRODUCIBILITY OF DISSSECTION PROCEDURE

Brain Region		Mean Weight mg ± S.E.M.
Medulla - Pons		262 ± 20
Cerebellum		327 ± 31
Cortex		756 ± 49
Midbrain		401 ± 40
Striatum		261 ± 25
Hypothalamus		61 ± 10

Fig. 9

Comm.

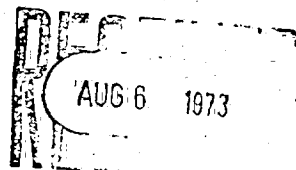
Dr. Bing
Dr. Gardner
Dr. Jacobson

PHARMACOLOGY

1
#662R1

THE COUNCIL FOR TOBACCO RESEARCH—U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8885



Application For Renewal of Research Grant:

(Use extra pages as needed)

First Renewal ☐

Second Renewal ☐

Date:
July 27, 1973

1. Principal Investigator (give title and degrees):

Herbert McKennis, Jr., S.B., Ph.D., Professor of Pharmacology.

2. Institution & address:

Department of Pharmacology
Medical College of Virginia
Richmond, Virginia 23298

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology, Medical College of Virginia; Department of Toxicology, Karolinska Institute, Stockholm; Department of Chemistry, Duke University, Durham, N. C.; others, as required.

4. Short title of study:

Biological Activity of Tobacco Smoke Components and Allied Substances.

5. Proposed renewal date: 9-30-73

6. How results to date have changed earlier specific research aims:

Original application stated that the specific aim of the project was to develop information on the role of nicotine metabolites and other substances in producing or altering the biological effects ordinarily ascribed to nicotine. Consistent with this goal, considerable new information has been developed. No change on research aims has become necessary, except in the use and development of analytical techniques, which are essentially only tools for completion of the project. It may be mentioned that during the course of investigations in this laboratory, dating back in inception many years, that there have been a number of changes in the public scientific conception of the role of nicotine metabolites in the producing or altering biological ef-

7. How results to date have changed earlier working hypothesis:

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chronic smokers and correlate much better with the effect of smoking on neuronal activity.

B) METHODS OF PROCEDURE

Experiments will be carried out on male rats with initial weights of 150-170 gms and male guinea-pigs with initial weights of 150 gms.

Administration of Nicotine. Animals (rats and guinea-pigs) will be treated with approximately 2.0 mg/kg/day/animal of nicotine alkaloid placed in the drinking water for varying lengths of time. This concentration will be used because it has been shown to be equivalent to the "two-pack-a-day" dose of nicotine (Wenzel et al., 1964, 18-20) and has been shown to produce pharmacological effects. The animals will be caged in groups according to their treatment. Four rats or two guinea-pigs will be placed in each cage. Under these circumstances it has been observed that the animals will receive an average rather than an exact daily dose of 1.0 or 2.0 mg/kg (Wenzel et al. 1964, 18).

Nicotine will be administered in an average concentration of 1.0 or 2.0 mg/100 ml water. The total volume of the nicotine solution to be administered will be kept slightly less than the volume of water which the group will consume in one day. This volume will be given at noon and untreated water will be made available the following morning after the nicotine solution has been consumed. It has been shown that this concentration of nicotine will not impart a taste to the water and the rats show no preference for the solution of nicotine or untreated water. Depending upon the results obtained other doses of nicotine will also be studied.

1003542160

At varying periods of time of treatment--2 wks., 1, 2, 3, 4, 5, 6, 8, 10, and 12 months--the animals will be killed and hearts and brains removed for determination of the effect of nicotine in releasing

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1003542120

14. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
None			

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
The action of calcium on secretion*	USPHS (NIH)	\$147,850.	1/1/74 - 12/31/78

* Any award from the NIH will be used to study the mechanism of ACTH action on the adrenal cortex.

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Check is payable to

Mailing address for check:

Principal investigator

Typed Name Ronald P. Rubin, Ph.D.

Signature Ronald P. Rubin Date July 15, 1973

Telephone 212 270-1356 -
Area Code Number Extension

Responsible officer of institution

Typed Name _____

Title _____

Signature _____ Date _____

Telephone _____
Area Code Number Extension

1003542131

William Schaffner, M. D. - Curriculum Vitae

MEMBERSHIP IN PROFESSIONAL SOCIETIES:

- 1.
- 2.
- 3.
- 4.
- 5.
- 6.
- 7.

REDACTED

REDACTED

REDACTED

LICENSE:

Tennessee

REDACTED

BOARD CERTIFICATION:

1. Diplomate of the National Board of Medical Examiners
2. Diplomate of the American Board of Internal Medicine
3. Diplomate in the subspecialty of Infectious Diseases
(American Board of Internal Medicine)

1003542196

Perfused Brain Slices . The rat brain will first be dissected into six regions according to a modification in the procedure described by Iversen and Glowinski (22). These include: medulla-pons; cerebellum; cortex; midbrain, striatum and hypothalamus. The reproducibility of the dissection procedure is depicted on Table 2 which shows the mean weight of each region \pm standard error of the mean. The endogenous norepinephrine of each region is depicted on Table 3. These determinations were carried out according to a modification of the automated trihydroxyindole procedure of Robinson and Watts (23). These values agree reasonably well with those obtained from the literature, so also serve as a measure of the reproducibility of the dissection technique.

Following the dissection technique the various brain regions will then be chopped with the McIlwain tissue chopper according to the procedure described by Ziance and Rutledge (24,25). The chopper is set at 0.3 mm and the brain tissue is chopped two times in two directions which are at right angles.

The tissue is then scrapped off the plastic disc into 12 ml centrifuge tubes with Krebs-Henseleit solution. The tissue is thoroughly suspended via a vortex blender. The suspension is then centrifuged in a clinical centrifuge at room temperature for two min. at 1,000 \times g. The pellet is then resuspended in physiological salt solution and incubated at 37°C for 10 min. in a shaking water bath. ^3H -1-norepinephrine (10 μe ; specific activity \sim 6 C/mmol; final concentration of norepinephrine will be 10^{-6}M) will then be added and the tubes incubated for 15 min. at 37° in a 95% O_2 - 5% CO_2 atmosphere. Similar concentration of labeled dopamine and serotonin will be used in experiments studying the release of these neurotransmitters. The total incubation

1003542162

2

6. How results to date have changed earlier specific research aims:

Continued...

fects attributed to nicotine. Following the initial isolation of many of the metabolites a viewpoint developed wherein the metabolites were accepted as being relatively inert. As more data has been generated in this laboratory and elsewhere, the viewpoint seems to shift to one in which the possible role of metabolites in producing some of the biological effects attributed to nicotine may be more important than originally conceived. Work that has been influential in this changing concept includes (a) the work of von Euler and collaborators, which shows an effect of nicotine isomethonium ion, in producing a delayed release of norepinephrine from the aorta, (b) the studies of Bost and McKennis, showing in the dog that the same nicotine metabolite, nicotine isomethonium ion, has a greater effect than does nicotine in increasing peripheral vascular resistance, and (c) the CTR-USA sponsored work of Essman showing an effect of two nicotine metabolites, cotinine and 3-pyridylacetate, on the consolidation phase of learning. Coupled with these and other studies on biological effects of nicotine, one now sees general confirmation of the fact that after smoking nicotine levels of the blood quickly approach zero, while the levels of cotinine (or apparent) are readily detectable for many days. This work from other laboratories, including those of Rand, has focused attention upon the possible role of cotinine in maintaining some of the feeling of satisfaction which smokers attribute to the use of tobacco. In turn this raises the question of whether or not the effects or possible effects attributed to cotinine arise from cotinine itself or a metabolite of cotinine. Essman has already noted that 3-pyridylacetate, a metabolite of nicotine via the chain nicotine \rightarrow cotinine \rightarrow γ -3-pyridyl- γ -oxo-N-methylbutyramide (allohydroxycotinine) has activity and is less active than cotinine in his studies. As will be seen later in this report, 3-pyridylacetate is further metabolized to N-3-pyridylglycine, a compound which has not been subjected to thorough biological studies. Additional gaps in the general picture included the fact that demethylcotinine, which arises from cotinine and can undergo metabolism to 3-pyridylacetate via the chain demethylcotinine \rightarrow γ -(3-pyridyl)- γ -oxobutyramide (a hypothetical intermediate) \rightarrow γ -3-pyridyl- γ -oxobutyric acid. 3-Pyridylacetate has been subjected only to limited studies on smooth muscle. 3-Hydroxycotinine, an additional metabolite of nicotine, has not been subjected to comprehensive biological studies. New methods (preliminary for the detection of many of these compounds) appear in this report, and studies on some of the biological effects of metan nicotine (*cis* and *trans*) are also included in this report. Possible gas-chromatographic analytical methods for metan nicotine are included in an abstract that accompanies this report.

The importance of chemical and physical techniques to confirm data from the radioimmunoassay procedures rapidly emerging from other laboratories resides in the fact that there is an underlying lack of specificity in radioimmunoassays. Those studies on radioimmunoassay procedures for nicotine and cotinine already brought to our attention do not include the full gamut of mammalian metabolites of nicotine, nor do they include a number of the congeners of nicotine known to be present in tobacco smoke.

1003542096

Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

Current

Title of Project	Source	Amount	Duration
Role of Cholinergic Agents in Adrenergic Transmission	National Institutes of Neurological Diseases and Stroke	62,970	Two yrs. 6-1-72 5-31-74

Pending

None

1003542181

11. SHORT BIOGRAPHICAL SKETCH OF PRINCIPAL INVESTIGATOR

Estimated percentage of time to be devoted to proposed work - 25%

NAME: Thomas C. Westfall REDACTED

TITLE: Associate Professor of Pharmacology

BIRTHDATE: REDACTED

PLACE OF BIRTH: REDACTED

NATIONALITY: REDACTED

EDUCATION:

West Virginia University, Morgantown, W. Va.,
A.B., 1959, Biology and Chemistry
West Virginia University, Morgantown, W. Va.,
M.S., 1961, Pharmacology
West Virginia University, Morgantown, W. Va.,
Ph.D., 1962, Pharmacology
Karolinska Institute, Stockholm, Sweden, Postdoc.,
1963-64 Neurochemical Pharmacology

HONORS:

Board of Governor Scholarship, West Va. Univ., 1955-59
National Institutes of Health Predoctoral Fellowship,
1959-62
National Institutes of Health Postdoctoral Award
(National Heart Institute) 1963-64

PRECEPTORS:

1959-62	Dr. Daniel T. Watts	Professor U.S. von Euler
	Dean of Graduate Studies	Chairman of Physiology
	Medical College of Virginia	Karolinska Institute
	Virginia Commonwealth University	Stockholm, Sweden

SOCIETIES:

REDACTED

REDACTED

MAJOR RESEARCH INTEREST:

1003542171

Influence of drugs on uptake, storage, release and
inactivation of biogenic amines.

1. Compounds to be tested

At present, we intend to study drugs related to nicotine in structure or behavior such as lobeline, cotinine, nicotinemethyliodide (a peripheral nicotine-like drug) and nornicotine. The above compounds were chosen because of their ready availability, and the fact that they might be the best ones to test such a screening procedure. While lobeline has been found to have no nicotine-like effect in this laboratory, this drug has not been completely studied. Furthermore, we would also like to study it in greater detail from the point of view of having effects of its own. In other words, instead of trying to determine whether the nicotine state will transfer to such compounds as a primary objective, we would also like to study each compound in terms of its own possible state dependent effect. This is of extreme importance in terms of attempting to find out what compounds in tobacco have behavioral effects of their own.

2. Overall Behavioral Approach

Compounds will be studied in Aprague Dawley male rats and male mice in various phases. These phases will include the following: (1) initial screening studies will be carried out in both passive and active avoidance procedures, 2) Analagous studies will be conducted in a similar population of rats, 3) Compounds having state dependent effects of their own [determined from 1) and 2)] will be studied in operant procedures involving rats [this will be accomplished by, a) determining whether the nicotine cue will transfer to such compounds, b) or whether rats can be trained to discriminate between the drug and non drug state], and 4) compounds will also be studied as antagonists in rats trained to discriminate between nicotine and saline. In these latter approaches a population of trained rats will be maintained to test a compound as to whether it has nicotine-like or antagonist properties. This will be an ongoing experiment and will involve several well trained animals.

1003542107

13 Budget: (1st year)

35.

A. Salaries (Personnel by names)
Professional

% time

Amount

Thomas C. Westfall

25%

REDACTED

Technical

Lab Specialist A
(Mary Brasted)

100%

REDACTED

Sub-Total

REDACTED

B. Consumable Supplies (list by categories)

Chemicals and Isotopes
Animals and Animal Care

1,500.

5,000.

Sub-Total

6,500.

C. Other Expenses (itemize)

Publications (400.)
Travel (300.)
Computer time(500.)

1,200.

Sub-Total

1,200.

D. Permanent Equipment (itemize)

Tissue Slicer

600.

1003542178

Sub-Total

600.

E. Overhead (15% of A+B+C)

2,785.

Total

REDACTED

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	6,500.	1,400	0	2,925	REDACTED
Year 3	REDACTED					

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Signature Thomas C. Westfall

Director of Project

Signature Ray C. Ford

Business Officer of the Institution

Telephone

Telephone

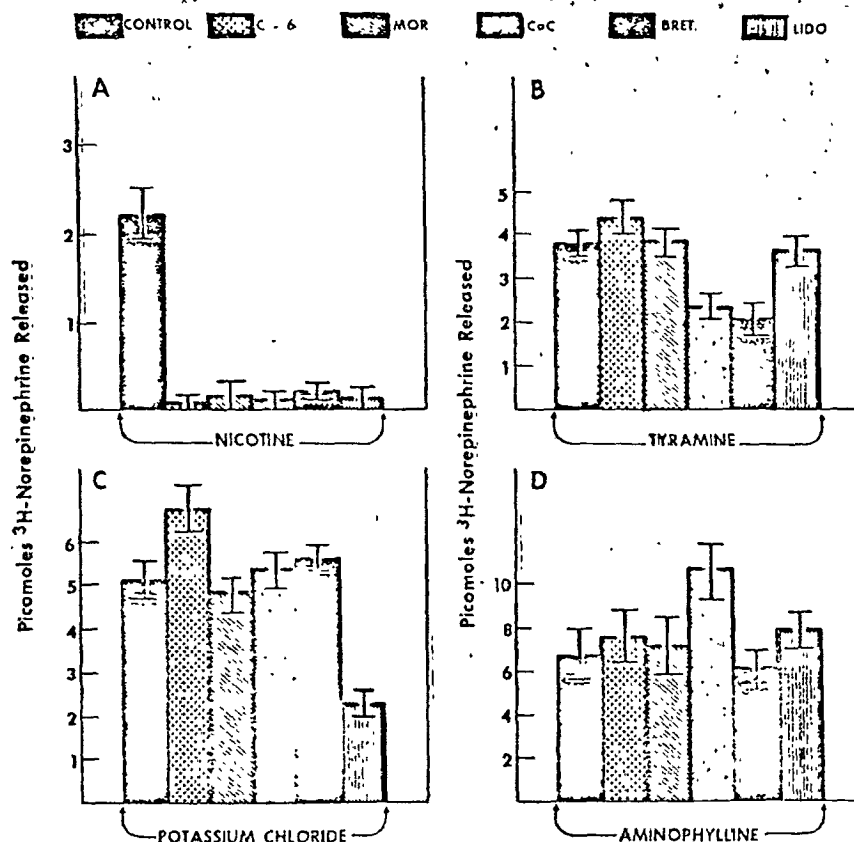


Fig. 1 This figure depicts the effect of nicotine 100µg (panel A), tyramine 300µg (panel B), potassium chloride .3M (panel C) and aminophylline 50mg (panel D) on the release of ^3H -norepinephrine from the perfused guinea-pig heart alone or in the presence of hexamethonium (C-6 10^{-5}M), morphine 3×10^{-4} , cocaine 10^{-5}M , bretylium 10^{-5}M and lidocaine 5×10^{-5} + S.E.M. I. It can be seen that all 5 drugs blocked the release of ^3H -NE to nicotine, while only cocaine and bretylium reduced the release by tyramine and lidocaine, the release by ^3H -NE by KCl. None of the drugs blocked the release by ^3H -NE aminophylline.

1003542149

it follows that studies are indicated to ascertain whether this action of nicotine, like ACTH, is mediated through cyclic AMP. Therefore, experiments will be carried out to discern whether the steroidogenic effect of exogenous cyclic AMP (or its more lipid soluble dibutyryl analogue) is also potentiated by nicotine. The general protocol of such experiments involves dispersing the adrenal cortical cells by exposure to trypsin; the cells are then removed from the trypsin - containing medium, suspended in various media for 1-2 hours. The cells are centrifuged and the supernatant assayed for steroid by protein binding assay.

If the action of nicotine involves cyclic AMP as an intermediate, then inhibition of the enzyme responsible for the breakdown of cyclic AMP should enhance the effects of nicotine on steroid production. Thus, experiments will also be conducted on isolated cortical cells to study how various inhibitors of phosphodiesterase influence the steroidogenic activity of nicotine.

Since the action of ACTH is associated with an increase in adrenal cyclic AMP levels, (Carchman *et al.*, 1971) nicotine might also be expected to augment cyclic AMP, if it acts by a mechanism which is similar to ACTH. Experiments are planned to discern whether this is indeed the case. The effect of nicotine on cyclic AMP levels and steroid production will be determined in isolated cortical cells with or without ACTH - in an attempt to establish a relationship - if one does, in fact, exist - between the enhancement of the ACTH response by nicotine and cyclic AMP concentrations.

B. Prostaglandins. Prostaglandins are a family of unsaturated fatty acids which are ubiquitously distributed and have a wide variety of metabolic and endocrine effects, including putative modulators of the secretory process. Many of the effects of prostaglandins are manifest in those systems where cyclic AMP is believed to mediate the response of the stimulating hormone, such as the adrenal cortex. Prostaglandins are present in the adrenal cortex and when exogenously administered they can mimic the action of ACTH in stimulating steroidogenesis in isolated cat cortical cells (Table I). The steroidogenic action of the prostaglandins may be related to an increase in the formation of cyclic AMP (Saruta and Kaplan, 1972) or they may produce their effects in the adrenal by causing a translocation of cellular calcium (Ramwell and Shaw, 1970).

In light of the potential importance of prostaglandins in the action of ACTH, the effects of nicotine on the steroidogenic activity of prostaglandin E₁ and E₂ will be investigated in the isolated cell preparation. Such studies may help to determine the physiological significance of the role of prostaglandins in ACTH action and may also aid in elucidating whether the mechanism of nicotine potentiation of ACTH-induced steroidogenesis involves the prostaglandins.

C. Studies on the intact gland. Finally, experiments are planned to investigate the action of nicotine on steroid production and release from the isolated perfused cat adrenal gland. Nicotine stimulates steroidogenesis in isolated adrenal cells, but greater significance can be attributed to this finding if it can be duplicated in the intact adrenal gland. Glands perfused with Locke's solution will be exposed to varying concentrations of nicotine for different time periods, in the absence and presence of ACTH. Steroid release will be measured by a previously published acid-fluorescence method (Jaanus *et al.*, 1970), after collecting the perfusate from a cannula placed in the adrenolumbar vein.

1003542133

#932 - HEINRICHS

1003542217

12. Summary Progress Report:

CTR Grant #869

Progress Report # 1
1/1/73 - 6/31/73

Name of Investigator: Ronald P. Rubin, Ph.D.
Name of Institution: State University of New York, Downstate Medical Center
Mailing Address: Department of Pharmacology, State University of New York,
Downstate Medical Center, 450 Clarkson Ave.
Brooklyn, New York 11203

Title of Grant: The Action of Nicotine on the Adrenal Gland.

Nicotine is an agent which enhances both catecholamine (Silvette et al., 1961) and corticosteroid secretion (Kershbaum et al., 1968) from human and animal adrenal glands. This agent stimulates the medullary chromaffin cells directly (Rubin and Miele, 1968) and indirectly via the hypothalamus (Silvette et al., 1961), however, the question as to whether the steroidogenic effect of nicotine is the result of a direct action on adrenal cortical cells or an indirect one via the hypothalamic-pituitary pathway has been a matter of debate. This project has been approached from the major perspectives: (a) To elucidate the mechanism by which nicotine, acetylcholine and other medullary secretagogues enhance catecholamine secretion and (b) to discern whether nicotine exerts a direct action on adrenocortical cells, and if so, by what mechanism.

Medulla. Cyclic AMP (Robison et al., 1971) and calcium (Rubin, 1970) are critical intermediates in the actions of many hormones. Previous work has already established that calcium is required for stimulation of medullary catecholamine release by nicotine (Douglas and Rubin, 1961a) as well as acetylcholine (Douglas and Rubin, 1961b). Other studies from our laboratory have shown that the cat adrenal gland contains significant concentrations of cyclic AMP (Carchman et al., 1971). Our initial investigations directly related to this project demonstrated that a portion of the cyclic nucleotide was contained in the medulla; for when the cortex and medulla were separated and cyclic AMP analysis carried out on both tissues the medulla was found to contain 20.7% (± 5.2) of the total cyclic AMP or 29.6 pmoles/gland (± 12.8) (mean of 3 experiments). Cyclic AMP was measured by the radioimmunoassay method of Steiner et al., 1969.

After establishing the presence of cyclic AMP in the cat adrenal medulla, experiments were designed to determine whether medullary secretagogues, such as nicotine and acetylcholine, could alter adrenal cyclic AMP concentrations, because if this cyclic nucleotide is a direct modulator of the secretory rate, then an increase in catecholamine release should be associated with an increase in adrenal cyclic AMP.

Cat adrenal glands were perfused in situ by a modification of the method originally developed in our laboratory (Douglas and Rubin, 1961b). The original technique was modified so that both glands could be perfused simultaneously; the left gland was used as the control and the right gland was exposed to nicotine or acetylcholine. Since paired adrenals within the same animal behave almost identically, using the left adrenal as the control gland eliminates the biological variability from cat to cat, which may obscure any small changes in cyclic AMP levels after stimulation. A similar approach was previously used in our laboratory to determine the effects of ACTH in cortical cyclic AMP levels (Carchman et al., 1971).

1003542134

THE ROCKEFELLER UNIVERSITY

NEW YORK, N.Y. 10021

May 9, 1973

Dr. Frederic W. Nordsiek
The Council for Tobacco Research - U.S.A., Inc.
110 East 59th Street
New York, N. Y. 10022

Dear Dr. Nordsiek:

At Dr. Neal Miller's suggestion, I have reviewed Dr. Thomas C. Westfall's proposal to the Council for Tobacco Research entitled "Action of Nicotine on Peripheral and Central Neurons in Animals Chronically Exposed to Nicotine."

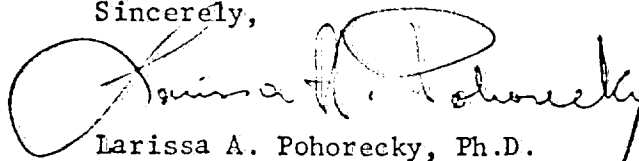
In my opinion, the proposal is well organized and well written. Dr. Westfall has sufficient knowledge and previous experience in the catecholamine field and in nicotine research to be able to conduct these experiments effectively. The results from the proposed experiments will contribute significantly to the knowledge on the chronic effects of nicotine in rodents.

The grant proposal provides good background information on the proposed experiments and on the literature in the field. Dr. Westfall's working hypothesis is scientifically sound and the experimental design is well directed toward the specific aims of the project. The length of time for the project, and the budget requested, appear to me quite adequate.

As far as the experimental design is concerned, I would suggest only two points. First, that it would be useful to include the determination of both monoamine oxidase and catechol-O-methyl transferase activities not only on liver and heart as described by Dr. Westfall, but also on the brain samples. It is very likely that, if the activities of these two norepinephrine-catabolyzing enzymes are altered by nicotine in peripheral organs (liver and heart) as stated in the proposal, a change in the activities of both enzymes might also occur in central noradrenergic neurons. This, in fact, might be quite significant in the adjustment of the central noradrenergic neurons to chronic nicotine exposure.

Secondly, in the experiments where the release of labeled amines will be examined in chopped brain slices, I think that it is very important to first examine whether there are any differences in the uptake of the labeled monoamines by the tissues obtained from animals exposed to nicotine chronically. Thus, the subsequently examined release of the labeled amines might be confounded by an unequal pre-labeling of the brain slices.

Sincerely,


Larissa A. Pohorecky, Ph.D.

LAP:emg

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10. Space and facilities available (when elsewhere than item 2 indicates, state location):

We have available a Varian 600-D gas chromatograph equipped with a flame ionization detector for use in this study. The University's Sigma 7 computer is more than adequate for our statistical needs, and we have programs currently in use which will take care of the data processing in the proposed project. An LKB-9000 combined mass spectrometer-gas chromatograph is available in the department for confirmation of compound identity. The science and medical libraries of the University are quite adequate for the needs of the project, and ample laboratory space is available.

11. Additional facilities required:

None

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

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IN VIVO OR IN VITRO REFLECT THE ADMINISTRATION OF NICOTINE IN HUMANS IN THE FORM OF TOBACCO SMOKING? It would appear that studies carried out after animals were chronically exposed to nicotine would come much closer in mimicing the human situation. There are in fact several observations from our own laboratory indicating differences between the acute and chronic administration of nicotine on adrenergic neuronal activity:

1) Injections of nicotine in divided daily doses results in an increase in the 24 hour urinary excretion of catecholamines but after 14 days of continued nicotine administration the elevated urinary catecholamine levels are normal. A study of the mechanism of the return to normal of the elevated urinary catecholamines after chronic administration revealed that there was a significant increase in the monoamine oxidase activity of the heart and liver and an increase in the catechol-o-methyl transferase activity of the liver. It was concluded that tolerance to nicotine induced elevations of urinary catecholamines was due to increased metabolic enzyme activity resulting in faster metabolism of the catecholamines released from the adrenal medulla and adrenergic nerve terminals (see the enclosed manuscript Biochem. Pharmacol. 20: 1627, 1971 (14)).

2) Acute injections of nicotine had no effect on the turnover of norepinephrine (a marker for adrenergic nerve activity) in the rat heart but following chronic daily administration for 47 days there was a significant increase in amine turnover (15) (see the enclosed manuscript Eur. J. Pharmacol. 10: 19, 1970). The mechanism of this difference between the behavior on norepinephrine turnover before and after chronic exposure to nicotine is not clear but certainly warrants further investigation.

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The addition of nicotine (in the concentration range of 2×10^{-5} - 10^{-4} M) to the fluid perfusing the right adrenal for 3-12 minutes produced an average increase in cyclic AMP levels of 79.3% (± 19.5). Similarly, exposure to acetylcholine (6×10^{-6} - 4×10^{-5} M) for 3-10 minutes augmented cyclic AMP levels by 65.5% (± 20.9) when the stimulated right gland was compared to its control left gland (Table II). Although nicotine and acetylcholine produced consistent increases in adrenal cyclic AMP levels when added to the perfusion medium in concentrations which greatly enhance catecholamine release, there was no obvious correlation between the concentration of secretagogue or exposure time and the increase in cyclic AMP levels (Table II).

Since perfusion with theophylline - an inhibitor of phosphodiesterase, the enzyme which degrades cyclic AMP - did not markedly enhance tissue adrenal cyclic AMP levels - we felt that perhaps a certain fraction of the cyclic AMP produced during stimulation was released into the perfusate along with the catecholamine. That this was indeed the case is shown in Table III. When the adrenal perfusate - collected by means of a polyethylene cannula placed into the adrenolumbar vein - was assayed for cyclic AMP, an increase in the release of cyclic nucleotide was readily demonstrable during exposure to nicotine or acetylcholine. Moreover, higher concentrations of secretagogue elicited higher rates of cyclic AMP release (Table III). Since the entire medulla at rest contains only about 30 pmoles cyclic AMP, the amount of cyclic nucleotide released during a 5-10 minute period of high secretory activity can approach or even exceed that which is initially present in the medulla.

These data thus demonstrate that although medullary stimulation by nicotine or acetylcholine is associated with an increase in adrenal cyclic AMP concentrations, it is difficult to attempt to relate tissue levels of cyclic nucleotide with secretory rates, since a significant proportion of the nucleotide is released into the perfusate along with the catecholamine. Experiments are now in progress to discern whether the rates of cyclic AMP release are better correlated - both temporally and quantitatively - with catecholamine secretion.

Cortex. The perfused cat adrenal gland perfused in situ is a useful test preparation since it approximates the situation in vivo yet eliminates the many neural and humoral influences which interact to modulate hormone release. However, with this preparation it is somewhat difficult to accurately evaluate the effects of various agents on the response to ACTH. Responses to ACTH last for an hour or more, and this preparation responds to increasing ACTH concentrations with a prolongation of enhanced corticosteroid release rather than a increase in the peak response. Thus, the difficulty in obtaining dose-response relationships in the intact gland prompted us to employ another preparation to investigate the effect of nicotine on ACTH-induced steroidogenesis.

A technique for isolating a single-cell system has recently been developed in our laboratory. Cats are anesthetized, the adrenal glands removed, cut into pieces and the cortical cells dispersed by treatment with trypsin by a modification of the method of Sayers *et al.*, (1971). The freed cells are then collected, suspended in various media, and steroid production measured by protein binding assay (Murphy, 1970). This preparation is very sensitive to ACTH - responding in a dose-related manner to as little as 12 μ U ACTH (Table I). In addition, exogenously administered cyclic nucleotide and prostaglandin (E_2) are effective steroidogenic agents in this system (Table I).

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CURRICULUM VITAE OF WILLIAM SCHAFFNER, II

49 NAME William Schaffner, II
 DATE OF BIRTH
 MARRIED
 CHILDREN
 PRESENT ADDRESS
 PRESENT POSITION Assistant Professor of Medicine, Director, Clinical Bacteriology
 Laboratory, Hospital Epidemiologist
 DEGREES B.S. 1957 Yale University
 M.D. 1962 Cornell University Medical College

INTERNSHIP, RESIDENCIES, FELLOWSHIPS, AND MILITARY SERVICE:

1. Intern in Medicine, Vanderbilt University Hospital 1962-63
2. Assistant Resident in Medicine, Vanderbilt University
 Hospital 1963-64
3. USPHS Postdoctoral Fellow in Infectious Disease,
 Vanderbilt University School of Medicine 1964-66
4. Epidemic Intelligence Service Officer of the National
 Communicable Disease Center, USPHS, Assigned to
 Rhode Island Department of Health; was Acting Chief,
 Division of Epidemiology 1966-68
5. Chief Medical Resident, Vanderbilt University Hospital 1968-69

ACADEMIC AWARDS, etc.:

1. Ford Foundation Scholar, Yale University 1953-57
2. Fulbright Fellowship to Albert-Ludwigs University,
 Freiberg, Germany 1957-58
3. New York City Health Research Council Summer Fellowship 1960
4. L.S.U. Student Fellow in Inter American Program in
 Tropical Medicine, Guatemala (2 months) 1962
5. USPHS Postdoctoral Fellowship 1964-66
6. Fellow, Fifth International Teaching Seminar on
 Cardiovascular Epidemiology, Singapore 1972

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

July 31, 1973

Grant Application No. 927
PHARMACOLOGY

To: The committee comprising Drs. Gardner, Jacobson and Sommers

Subject: David J. Wilson, Ph.D., Vanderbilt University
New application No. 927
"Nicotine Levels in Human Milk"

History

This proposal was Case No. 203, and application was encouraged.

Application No. 927 requests \$8,973 for one year only.

Documents Submitted (attached)

1. Application dated July 25, 1973.
2. "DDT Concentrations in Human Milk", by Wilson et al., Am J Dis Child 125, 814 (1973).



F.W.N.

FWN:wg
Encls.

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Schechter, M.D. and J.A. Rosecrans: Nicotine as a discriminative stimulus in rats depleted of norepinephrine or 5-hydroxytryptamine. *Psychopharmacologia* 24: 417, 1972.

Schechter, M.D. and J.A. Rosecrans: Effect of mecamylamine on discrimination between nicotine- and arecoline-produced cues. *European J. Pharmacol.* 17: 179, 1972.

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8. Any additional facilities now required? Describe briefly:

No immediate requirement for additional facilities is now seen. Additional requirements, if any, may develop as new results are obtained.

9. Any changes in personnel? Append biographical sketches of new key professional personnel:

No changes in key professional personnel are contemplated. Those now engaged in the project are experienced and indicate an eagerness to complete various phases of the project now under study.

10. Append outline of experimental protocol for ensuing year. (See appendage.)

11. List publications or papers in press resulting from this or closely related work. (append reprints or manuscripts not previously sent).

See #12.

12. Summary progress report (append in standard form as separate document, unless recently submitted).

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#787B - FRIEDMAN

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PAPERS IN PRESS OR IN PREPARATION:

Dailey, J. W. and Westfall, T. C. The effects of adrenalectomy and adrenal steroids on the synthesis of norepinephrine in the rat. *J. Pharmacol. Exp. Ther.* (In Press) 1973.

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Atuk, N. O., Westfall, T. C. and Westfall, V. Altered catecholamine metabolism in recurrent jaundice evidence for catechol-o-methyl transferase deficiency.

Brase, D. A. and Westfall, T. C. Studies on the mechanism of stimulation of phenylalanine hydroxylase activity by short chain alcohols. *Biochem. Biophys. Acta.*

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Over the last couple of years we have been investigating the effect of nicotine on adrenergic nerve terminals using the isolated perfused guinea-pig heart, prelabeled with ^3H -norepinephrine as a model (See data submitted with application of August, 1971 and the enclosed reprint (13)). We have observed that nicotine produces an explosive increase in the efflux of ^3H -norepinephrine (release) from the perfused heart and it is the released amine which produces the sympathomimetic pharmacological effects (positive inotropic and chronotropic activity). There is strong evidence that the mechanism of this action is due to activation of a receptor (13) (see enclosed reprint) located on the axonal membrane of the adrenergic nerve plexus. The effect has an absolute requirement for extracellular Ca^{++} , and can be selectively blocked by pharmacological agents which will not block the release of norepinephrine by other drugs such as tyramine, KCl and aminophylline (Fig. 1,2; Table 1) (13).

Recently we have been able to demonstrate a release of norepinephrine from chopped brain tissue incubated with labeled norepinephrine (Fig. 3) as well as superfused brain tissue (Fig. 4,5,6). It is thought that many of the effects of nicotine on the central nervous system are due to the release of norepinephrine and other neurotransmitters.

A release of ^3H -NE has so far been observed from the rat hypothalamus, cortex, medulla-pons and cerebellum (Fig. 3,4). The effect is dependent upon extracellular Ca^{+2} and the release is blocked by hexamethonium and acetylcholine (Fig. 5).

Although these studies are quite important in defining the biochemical and molecular mechanism of action of nicotine, a question of paramount importance is: TO WHAT EXTENT DOES THE ACUTE ADMINISTRATION

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Educational Activities at MCV - Additional Activities (continued)

College of Virginia Methadone Clinic, conduct independent research projects, and participate in a weekly seminar series. These students are in the second summer of the program.

University Committees

Dr. Rosecrans is a member of the following committees: Student Evaluation of Faculty (Graduate School), Research Advisory Committee (Graduate School), and Drug Education Curriculum Committee (Universities).

Abstracts of Papers Presented at Scientific Meetings

Rosecrans, J.A., H.W. Youngken, Jr. and J.J. DeFeo: A pharmacological investigation of Valerian officianlis, Linne. A.Ph.A. Convention, Washington, D.C., 1960.

Rosecrans, J.A.: Effects of route of administration and means of feeding on the toxicity of chronic reserpine administration in young and old rats. A.A.A.S. Meetings, Montreal, 1964.

Guarino, A.M., J.A. Rosecrans and J.J. DeFeo: The interrelationships between chronic isolation stress and drug administration in male albino rats. A.A.A.S. Meetings, Montreal, 1964.

Rosecrans, J.A., A.T. Dren and E.F. Domino: Effects of physostigmine on rat brain acetylcholine, acetylcholinesterase and avoidance behavior. Fed. Proc. 25: 409, 1966.

Lovell, R.A., J.A. Rosecrans and D.X. Freedman: Effects of LSD on rat brain serotonin metabolism, Fed. Proc. 26: 240, 1967.

Rosecrans, J.A. and M.H. Sheard: Effects of an acute stressor on brain amine levels of normal and CNS lesioned rats. Pharmacologist 9: 224, 1967.

Rosecrans, J.A.: Effects of an acute stressor on rat brain serotonin metabolism. Fed. Proc. 27: 540, 1968.

Rosecrans, J.A. and S.F. Bernstein: Studies on the relationships between emotionality behavior and pituitary-adrenal function. Fed. Proc. 28: 580, 1969.

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PHARMACOLOGY

H927

Comm.

Dr. Gardner
Dr. Jacobson
Dr. Sommers

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

JUL 27 1973

Application for Research Grant

(Use extra pages as needed)

Date: JUL 25 1973

1. Principal Investigator (give title and degrees):

David J. Wilson, Ph.D., Professor of Chemistry

William Schaffner, II, M.D., Assistant Professor of Medicine

2. Institution & address:

Department of Chemistry

Vanderbilt University

Nashville, Tennessee 37235

3. Department(s) where research will be done or collaboration provided:

Department of Chemistry

Department of Medicine

4. Short title of study:

Nicotine Levels in Human Milk

5. Proposed starting date: 1 October 1973

6. Estimated time to complete: 12 months

7. Brief description of specific research aims:

The objective of this work is to determine the extent to which nicotine occurs in the milk of smoking nursing mothers.

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The working hypothesis of the study is that the effects on nursing rats of dosing the mothers with nicotine is due to transmittal of nicotine through the milk and that similar transmittal may be taking place in humans.

9. Details of experimental design and procedures (append extra pages as necessary)

It is well established that a number of drugs administered to nursing mothers are excreted in their milk; Catz and Giacoia cite some 84 references in their review on the subject.¹ It is also well established that smoking by pregnant women has a number of effects upon the fetus -- the newborn infants tend to be smaller than normal and there appears to be an increase in abortions and stillbirths.^{2,3,4,5,6,7}

The lethal dose of nicotine for an adult is roughly 60 mg; a smoker typically absorbs 2-3 mg of the alkaloid per cigaret. Nicotine is deactivated in the liver and excreted via the kidneys. In small doses it affects the cholinergic synapses, causes release of adrenaline, and shifts the brain's EEG toward an arousal pattern.⁸ Although nicotine in human milk is mentioned in the literature from time to time,^{9,10,11} there does not seem to be any appreciable amount of data available on the subject. A recent study on the effects on nursing rats of dosing the mother rats with small quantities of nicotine indicated that the drug (or possibly a toxic metabolite) was transmitted to the young rats with deleterious effect.¹² It is difficult to relate this study to possible effects on nursing human infants of smoking mothers, but its findings are thought-provoking.

We therefore propose to analyze approximately 50 samples of human milk for nicotine. Of these, a small (5-10) control group will be obtained from non-smokers, with the rest coming from light, moderate, and heavy smokers. About half of these samples are already available, left from a study on DDT levels which we recently completed; the remainder will be solicited from La Leche League, a women's organization concerned with breast feeding which was very helpful in providing samples and helpful suggestions for our DDT project and current work which we are doing on lead levels in human milk. A questionnaire will be used to obtain information about smoking and dietary habits, age of mother and infant, parity of mother, etc.

(see continuation page)

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JUSTIFICATION OF BUDGET

1. Personnel

Principal Investigator. Dr. T.C. Westfall has had considerable experience in the field of neurotransmitter synthesis, storage, release and metabolism and in conducting experiments at the tissue and biochemical level. He will direct and coordinate the various phases of the proposed research and will dedicate about 25% of his time to it. The salary support requested for Dr. T.C. Westfall is less than the amount represented by his per cent of effort to be devoted to this project. The differences will be applied to the University Cost Sharing Commitment.

Laboratory Specialist. The salary requested is for Miss Mary Brasted who has been working in Dr. Westfall's laboratory for three years. She is very experienced and extremely competent in conducting studies on perfused organs and isolated tissues. She will be responsible for treating the animals and in carrying out the various measurements as described in the methods.

2. Equipment

Only one piece of permanent equipment is being requested, that of a MacIlwain tissue slicer. This item is necessary to prepare all the brain slices. We are currently borrowing such an instrument.

3. Supplies

This constitutes the other major individual item in the budget and includes animal costs and care, chemicals, isotopes and glassware.

- a) Animals and Animal Care. This item is necessary because of the anticipated and calculated cost of the large numbers of rats and guinea-pigs which will be used to successfully complete this project. Guinea-pig cost \$5-6.00 each, \$0.12/day for care and rats cost \$3.00 each, \$0.06/day for care.
- b) Chemicals, Isotopes. The biggest item here will be the cost of isotopes, which will be used for the project, including 1-³H-norepinephrine \$120.00/1 mCi; ³H-dopamine, \$70/1 mCi; ³H-serotonin \$105.00/1 mCi. In addition there will be a fairly large amount of chemicals necessary.

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9. Details of experimental design and procedures (continued)

Nicotine will be determined in the samples by means of a modification of the gas chromatographic technique adapted by Burrows and coworkers¹³ from a method due to Schievelbein and Grundke.¹⁴ This method is capable of determining nicotine levels in the nanogram/nl range, and is much more sensitive than gas chromatographic methods for nicotine in urine.^{15,16} The sample (10 ml) is made alkaline and steam-distilled, and the distillate is made alkaline and extracted with methylene chloride. This extract is cleaned up on an alumina column; nicotine is eluted with 1-1 methylene chloride-ethyl alcohol, and this solution is chromatographed (8% carbowax 20 M + 2% KOH on Chromosorb W, acid washed and treated with hexamethyldisilazane) at 150°C, using a flame ionization detector.

BIBLIOGRAPHY

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- 10 H. Schievelbein, *Beitr. Tabakforsch.* 6, 199 (1962).
- 11 Ref. 7, Suppl. 1, p. 206.
- 12 R. F. Becker and J. C. Martin, *Am. J. Obstet. and Gynecol.* 110, 522 (1971).
- 13 T. E. Burrows, P. J. Corp, G. C. Jackson and B. F. J. Page, *Analyst (London)* 96, 81 (1971).
- 14 H. Schievelbein and K. Grundke, *Fresenius' Z. Analyt. Chem.* 237, 1 (1968).
- 15 A. H. Beckett and E. J. Triggs, *Nature* 211, 1415 (1966).
- 16 N. L. McNiven, K. H. Raisiughani, S. Patashnik, and R. I. Dorfman, *Nature* 208, 788 (1965).

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39. "An Outbreak of Pseudomonas cepacia Infection Due to Contaminated Anesthetics," W. Schaffner, G. Reisig, and R. A. Verrall, (submitted for publication).

ABSTRACTS:

1. "Lysostaphin: An Enzymatic Approach to the Therapy of Experimental Staphylococcal Infections," W. Schaffner, M. A. Melly, and M. G. Koenig, Clin. Res., 14, 343 (1966).
2. "An Outbreak of Sepsis Due to Contaminated Intravenous Fluid: Clinical, Epidemiological and Laboratory Observations," W. Schaffner, S. K. Felts, M. A. Melly, and M. G. Koenig, Ann. Int. Med., 76, 872 (1972).

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Hsu, C-Y and Westfall, T. C. Release of ³H-norepinephrine by aminophylline in the perfused guinea-pig heart.

Westfall, T. C. and Brasted, M. Specificity of blockade of the nicotine-induced release of norepinephrine from adrenergic neurons by various pharmacological agents.

PUBLISHED ABSTRACTS DURING THE PAST SEVEN YEARS:

1. Peach, M. J. and Westfall, T. C. Action of angiotensin on myocardial catecholamines in the rabbit. Fed. Proc., 24: 488, 1965.
2. Westfall, T. C. Influence of pronethalol, propranolol and iproveratril on uptake and storage of norepinephrine. Fed. Proc., 25: 260, 1966.
3. Westfall, T. C., Fleming, R. M., Fudger, M. K., and Clark, W. G. Effect of nicotine and related substances on amine levels in the brain. Symposium on The Effect of Nicotine and Smoking on the Central Nervous System. N.Y. Acad. Sci., April, 1966.
4. Westfall, T. C. Uptake and storage of norepinephrine following the administration of three beta adrenergic antagonists. Va. J. Sci., 17: 354, 1966.
5. Westfall, T. C. Influence of nicotine on catecholamine metabolism. Symposium Abstract. Tobacco and Health. Amer. Med. Assn., November, 1966.
6. Westfall, T. C. Influence of beta adrenergic antagonists on norepinephrine content in rat heart before and after NE infusion. Fed. Pro., 26: 569, 1967.
7. Westfall, T. C. Influence of beta adrenergic blockers on norepinephrine storage in the perfused guinea pig-heart. Va. J. Sci., 18: 202, 1967.
8. Westfall, T. C. The effect of beta adrenergic blocking drugs on the norepinephrine level in the perfused guinea-pig heart following NE infusion. The Pharmacologist, 9: 249, 1967.
9. Westfall, T. C. and Osada, H. Influence of adrenalectomy on the depletion of norepinephrine following treatment with metaraminol or guanethidine. Fed. Proc., 27: 601, 1968.
10. Moore, W. C. and Westfall, T. C. The influence of monoamine oxidase inhibitors on the accumulation of norepinephrine in reserpine treated rats. Va. J. Sci., 19: 206, 1968.
11. Westfall, T. C. and Osada, H. Influence of adrenalectomy on the turnover of norepinephrine in the rat heart. The Pharmacologist, 10: 158, 1968.

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SIGNIFICANCE OF THIS PROPOSAL

Nicotine is one of the most important and active ingredients in tobacco. Because of the wide spread use and continual implications of smoking as a health hazard, it is important to have a thorough understanding of the action of this drug on the nervous system. Studies of the acute administration of nicotine have been important in demonstrating the many actions of nicotine. A question of paramount importance however is: TO WHAT EXTENT DOES THE ACUTE ADMINISTRATION OF NICOTINE IN VIVO OR IN VITRO REFLECT THE ADMINISTRATION OF NICOTINE IN HUMANS TAKEN IN THE FORM OF TOBACCO SMOKING? Since smokers are actually chronic users of tobacco, it would appear that studies testing the effect of nicotine on tissues obtained from animals chronically exposed to nicotine would come much closer to mimicing the human situation. This is what we plan to do in this study. We have several very accurate ways of measuring the effect of nicotine on nervous tissue and therefore feel it will be of great importance to make these measurements on tissues taken from animals exposed to nicotine for varying periods of time. We feel this will give us a more accurate picture of what effect nicotine has on nervous tissue of man when it is administered from tobacco smoke. We feel that we will then be able to correlate the effects of nicotine with tobacco smoking in a much more valid way. It appears that up to the present time these studies have not been done so we really don't know what effects nicotine has on synaptic transmission, transmitter turnover, etc. We have reason to suspect that the effects of nicotine on neuronal function might be quite different when studied after animals have been chronically exposed to this alkaloid. We feel, therefore, that the studies suggested in the present proposal are quite important and will provide us with extremely valuable information.

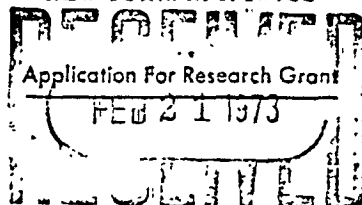
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Comm.

Dr. Bing
Dr. Gardner
Dr. Jacobson

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 30TH STREET
NEW YORK, N. Y. 10022



Date: February 1, 1973

1. Name of Investigator(s): (include Title and Degrees)

Thomas C. Westfall, A.B., M.S., Ph.D.
Associate Professor of Pharmacology

2. Institution &
Address:

Department of Pharmacology
University of Virginia School of Medicine
Charlottesville, Virginia

3. Short Title of Project: Action of Nicotine on Peripheral and Central Neurons
In Animals Chronically Exposed to Nicotine.

4. Proposed Starting Date: March 1, 1973

5. Anticipated Duration of this Specific Study: Two Years

6. Brief Description of Objectives or Specific Aims:

The main objective of this study is to compare the effect of nicotine on several parameters of neuronal activity when administered to naive preparations (tissues obtained from animals not previously exposed to nicotine) or tissues obtained from animals which have been constantly exposed to nicotine for varying lengths of time. The parameters to be measured are: a) release of norepinephrine from peripheral adrenergic neurons (perfused heart preparation), b) release of norepinephrine, dopamine or serotonin from central neurons (perfused brain slice preparation), c) turnover of norepinephrine, dopamine or serotonin and d) monoamine oxidase activity and catechol-O-methyl transferase activity. The study is based on the fact that we have very reliable and reproducible methods for measuring these effects and that smokers are constant users of tobacco. By comparing the effect of nicotine on tissues obtained from animals which have not been previously exposed to nicotine with those that have been exposed for varying periods of time we should have a more valid means of correlating the effect of nicotine on the nervous system in smokers and non-smokers.

7. Give a Brief Statement of your Working Hypothesis:

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12. Westfall, T. C. Influence of nicotine on the turnover of norepinephrine in brain and heart. J. Amer. Med. Assn., 1968.
13. Atuk, N. O., Westfall, T. C. and Donaldson, M. H. Catecholamine metabolism and alpha-receptor response to norepinephrine in familial pheochromocytoma. Clin.Res., 17: 57, 1969.
14. Westfall, T. C. and Brase, D. A. Adrenal stimulation and monoamine oxidase activity following daily nicotine treatment. Fed. Proc., 28: 287, 1969.
15. Atuk, N. O., Westfall, T. C. and Donaldson, M. H. Mechanism of normal blood pressure in familial pheochromocytoma. Proceedings of Am. Coll. of Physicians, 129-130, 1969.
16. Westfall, T. C. The effect of nicotine on the synthesis, uptake and metabolism of catecholamines. Proceedings of the Fourth International Pharmacol. Congress, Basel. July: 153, 1969.
17. Colombini, C., Westfall, T. C., and McCoy, E. The effect of LSD-25 on vitamin B-6 metabolism and brain amine levels in mice. Proc. Second International Meeting of Neurochem., Milan: 133, 1969.
18. Colombini, C., Westfall, T. C. and McCoy, E. Effects of LSD-25 and marijuana on vitamin B-6 synthesis and distribution in the mouse. Proc. Southeastern Sect. Amer. Chem. Soc., 1969.
19. Dailey, J. W. and Westfall, T. C. Effect of adrenal steroids on the turnover of H³-norepinephrine in the rat heart. Fed. Proc., 29: 413, 1970.
20. Westfall, T. C. and Peach, M. J. Influence of equilibration perfusion duration on H³-norepinephrine uptake and intracellular cation concentration in isolated guinea-pig hearts. Pharmacologist, 12: 234, 1970.
21. Dailey, J. W. and Westfall, T. C. Influence of adrenalectomy and steroid replacement on norepinephrine biosynthesis. Va. J. Sci., 21: 144, 1970.
22. Colombini, C., Westfall, T. C. and McCoy, E. The changes in Vitamin B-6 and brain amine metabolism in mice chronically treated with 9-tetrahydrocannabinol. Proceed. of International Psychopharmacol. Congress. Prague, August, 1970.
23. Atuk, N. O. and Westfall, T. C. The occurrence of hypertension in benign recurrent interhepatic cholestases. Clin. Res., 19: 80, 1971.
24. Westfall, T. C. Interaction of nicotinic and antinicotinic agents on heart rate and uptake of norepinephrine. Fed. Proc., 30: 446, 1971.

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In addition to these two observations from our own laboratory with nicotine there are many examples in the literature regarding differences between the acute and chronic administration of other drugs which effect neuronal transmission processes. For instance:

1) The psychoactive drug methamphetamine produces an increase in the concentration of serotonin in most areas of the brain following acute administration, with a decrease in the hypothalamus and cortex. With chronic administration, however, the serotonin content in the caudate nucleus is actually increased--rather than decreased (16).

2) The acute administration of imipramine and protriptylline (2 tricyclic antidepressant drugs) produces a decrease in the turnover of norepinephrine in the brain with no changes in the endogenous content of the amine. The chronic administration of these 2 drugs, on the other hand results in an increase in the turnover of norepinephrine and a decrease in the endogenous norepinephrine content (17).

Another observation made in our laboratory deserves special mention. We have been studying the release of ^3H -NE from the perfused rabbit heart in much the same manner as the guinea-pig heart mentioned above. If hearts are obtained from rabbits chronically treated with morphine in a fixed dose of 15 mg/kg/day for 35 days or in gradually increasing doses (up to 90 mg/kg/day) it has been observed that there is a greater release of ^3H -NE following nicotine administration in these hearts as compared to controls (Fig. 6). This demonstrates that the chronic administration of this drug (morphine) results in a quantitatively different response of the adrenergic nerve terminals to nicotine.

Since it is very clear that these may be marked quantitative and

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biochemical procedures to be utilized in this proposal. In many instances, the various publications include the techniques which have been cited. He spends three hours per week in teaching, instruction and administrative duties.

4. Affiliations

Dr. Weltman is a member of the

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In the past, Dr. Weltman and members of the research team of the Laboratories for Therapeutic Research have published investigations involving tranquilizing agents, hallucinogenic compounds (LSD-25, mescaline), audiogenic-seizure susceptibility, auditory stress, vibration stress and whirler mice, etc. These studies have been concerned with behavioral, biochemical, body growth and endocrinal effects produced by the various pharmacological agents, stress or mutant characteristics. Dr. Weltman has assisted Dr. Shirley D. Kraus periodically in teaching the Physiology course at Brooklyn College of Pharmacy. An integral part of the Physiology Laboratory is devoted to study of the effects of pharmacological agents (i.e., epinephrine and acetylcholine) on systolic blood pressure of rats using a Physiograph 6 Model. Representative publications by Dr. Weltman follow:

1. Sackler, A.M., Weltman, A.S. and Sackler, R.R.: Effects of Tranquilizing Agents on the Resistance of Rats to Histamine Stress. *Nature* 183:896-897, 1959.
2. Jurtshuk, P., Jr., Weltman, A.S. and Sackler, A.M.: Biochemical Responses of Rats to Auditory Stress. *Science* 129:1424-1425, 1959.
3. Sackler, A.M., Weltman, A.S., Bradshaw, M. and Jurtshuk, P., Jr.: Endocrine Changes Due to Auditory Stress. *Acta Endocrinologica* 31:405-418, 1959.
4. Sackler, A.M., Weltman, A.S., Bradshaw, M. and Neilman, F.: The Effects of Reserpine on Histamine Tolerance and Endocrine Organs of the Rat. *Acta Endocrinologica* 34:619-626, 1960.
5. Sackler, A.M., Weltman, A.S. and Jurtshuk, P., Jr.: Endocrine Aspects of Auditory Stress. *Aerospace Medicine* 31:749-759, 1960.
6. Sackler, A.M., Weltman, A.S. and Jurtshuk, P., Jr.: Effects of Splenectomy on the Resistance of Rats to Histamine Stress. *Nature* 190:274, 1961.
7. Weltman, A.S., Sackler, A.M. and Gennis, J.: Effects of Handling on Weight Gains and Endocrine Organs in Mature Male Rats. *J. Applied Physiology* 16:587-588, 1961.
8. Sackler, A.M. and Weltman, A.S.: Endocrine and Behavioral Aspects on Intense Auditory Stress, p. 255-283 in *Psychophysiologie, Neuropharmacologie et Biochemie de la Crise Audiogene*, Centre National de la Recherche Scientifique, Paris, 1963.
9. Weltman, A.S. and Sackler, A.M.: Effects of Thymectomy on the Resistance of Rats to Drowning and Histamine Stress. *Nature* 192:460, 1961.
10. Weltman, A.S., Owens, H. and Sackler, A.M.: Effects of Age and Thyrectomy on Urinary 17-Ketosteroid Levels in Male Rats. *Nature* 194:1087-1088, 1962.

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7. Changes or Additions to Experimental Design and Procedures: (Attach Separate Pages)

None except as mentioned in Section 5 above.

8. Additional Requirements:

None

9. Changes in Personnel with Biographical Sketches of new Personnel (append):

None

10. Publications or Papers in Press resulting from the Project or closely related work

Please see attached Progress Report #3.

³H-norepinephrine from the perfused heart or brain.

Perfused Heart Preparation. Hearts will be removed from the animals (guinea-pigs and rats) under pentobarbital anesthesia and immediately connected to an Anderson-Craver coronary perfusion apparatus (Metro Scientific Co.) via the aorta. The normal perfusion medium contains in millimoles per liter: NaCl, 119.8; KCl, 5.63; CaCl₂, 2.16; MgCl₂, 2.10; dextrose, 100 and NaHCO₃, 25.0. The solution will be bubbled with 95% O₂ - 5% CO₂; temperature maintained at 37 ± 1°C and pH at 7.32 to 7.45. All hearts will be perfused at a constant flow of 6.0 ± .5 ml/min. with a Harvard perfusion pump. Following an equilibration period the hearts will be perfused with 1.0 ng/ml of 1-³H-norepinephrine for 20 minutes to label the endogenous store. The hearts will then be switched to a norepinephrine free-medium and the perfusate effluents continuously collected and analyzed. After 10-20 min. of perfusion with a norepinephrine-free medium nicotine in various concentrations will be administered via a side arm cannula.

Analysis of ³H-norepinephrine. The perfusate effluents will be collected in graduated tubes containing ascorbic acid (5 mg). ³H-norepinephrine will then be analyzed by liquid scintillation spectrometer following alumina column chromatography as described in Westfall and Osada (1969, 21) and Westfall and Brasted (1972, 13). For liquid scintillation counting 1.0 ml of sample will be placed in 10 ml of Triton-based solution containing 5.5 g of 2,5-diphenyloxazole (PPO); 150 mg of 1,4-bis [2-(5-Phenyloxazolyl)]-Benzene (POPOP) and 2:1 mixture of toluene and Triton X-100 and counted in a Packard Tri-Carb liquid scintillation spectrometer. Counting efficiency as determined by external standardization is 18-20%.

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12. Biographical sketches of investigator(s) and other professional personnel.

13. Publications.

CURRICULUM VITAE OF DAVID J. WILSON

We have available a Varian 600-B gas chromatograph equipped with a flame

EDUCATION:

B.S. 1952 Stanford University, Stanford, California
Ph.D. 1958 California Institute of Technology, Pasadena, California

SCIENTIFIC EXPERIENCE:

1. Stanford University (1952-53) - National Science Foundation Fellow in chemistry, research on the thermal decomposition of nitrogen pentoxide in the presence of nitric oxide, under Dr. H. S. Johnston.
2. Army Chemical Center, Maryland (1953-55) - Physical sciences assistant, Analytical Branch, Chemical Division, Chemical and Radiological Laboratories. Director; Mr. Sam Sass. Analytical research and routine analyses connected with organic phosphonates.
3. Stanford University (1955-56) - National Science Foundation Fellow in chemistry, research on the thermal decomposition of nitryl chloride, on the computation of pre-exponential factors in gas-phase reactions, and on the isotope effect in the oxidation of carbon monoxide by nitrogen dioxide. This work was done under the direction of Dr. H. S. Johnston.
4. California Institute of Technology (1956-57) - National Science Foundation Fellow in chemistry, research on the thermal decomposition of nitryl chloride and on temperature gradients in reaction cells, under Dr. H. S. Johnston.
5. University of Rochester (1957-69) - Instructor (1957-60), assistant professor (1960-63), associate professor (1963-67), and professor of chemistry, research on the theory of energy transfer processes in gas reactions, on the sensitized photodecomposition of nitryl chloride, in nuclear magnetic resonance, and in the quantum theory of inelastic scattering; undergraduate and graduate instruction in chemistry; section editor, Chemical Abstracts (1958-62); Alfred P. Sloan Fellow (1964-66). Visiting Senior Lecturer, University of Ife, Nigeria (1964-65).
6. Vanderbilt University (1969-Present) - Professor of chemistry; research in gas reactions and energy transfer processes in gases, investigation of pesticide and heavy metal residues, foam flotation methods, undergraduate and graduate instruction in chemistry.

PROFESSIONAL SOCIETIES:

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Curriculum Vitae
W.L. Heinrichs, M.D., Ph.D.

Page 4

Organization Responsibilities:

Program Committee Member	Washington State Obstetrical Association	1969-
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School of Medicine Committees:

Subject Committee	Embryology and Tissues Structures	1968-69
Advisory Committee	E-1970 Medical Students	1970-

University Hospital Committees:

Member	Infection Committee	1968-70
Member	Internship Interviewing	1969-70
Member	Perinatal Mortality	1968-69
Member	Scientific Advisory Board Clinical Research Center	1969-

1003542233

Plans for the Future

In section 5 of the attached renewal proposal we indicate the major direction in which the study is heading. While we have plenty of work left to do on our cross-sectional tabulations we propose to move more in the direction of longitudinal analyses, over longer time periods when possible. We have focussed primarily on characteristics associated with the presence or absence of smoking. We should begin to devote more attention to characteristics associated with starting and stopping smoking or other changes in smoking habits. For example, we would like to determine whether the psychological questionnaire items which best differentiate smokers from non-smokers also differentiate non-smokers who will start smoking from non-smokers who remain non-smokers.

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Curriculum Vitae
W.L. Heinrichs, M.D., Ph.D.

Page 3

Professional Organizations:

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Participation in Symposia or
International Scientific Conferences:

Ghent, Belgium	2nd Symposium on Steroid Hormones: "Androgens in Normal and Pathological Conditions."	1965
Princeton, New Jersey	Macy Conference on Research and Education in Obstetrics and Reproduction.	1967
Rome, Italy	4th Meeting of the International Study Group for Steroid Hormones.	1969

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8. Brief statement of working hypothesis: Nicotine and acetaldehyde are both present in appreciable quantities in cigarette smoke. The scientific literature contained in Tobacco: Experimental and Clinical Studies (Larson, Haag, and Silvette, 1961; Larson and Silvette, 1968; Larson and Silvette, 1971; Larson and Silvette, in preparation) provides ample evidence for the conclusion that nicotine and acetaldehyde depend upon the release of endogenous norepinephrine from sympathetic nerve endings and the adrenal medulla for their cardiovascular actions. It is of interest to compare the mechanism of sympathomimetic action of these two agents at the cellular level and in the cardiovascular system of an animal model in order to determine whether acute exposure to both agents simultaneously enhances the cardiovascular effects. Information generated by this investigation will contribute to an understanding of potential importance of the interactions of nicotine and acetaldehyde in the cardiovascular action of cigarette smoke.

Our laboratory has recently discovered that acetaldehyde could partially reverse the adrenergic neurone blockade induced by guanethidine in an isolated smooth muscle preparation (Lai and Hudgins, Pharmacologist, in press). In the light of this finding it appears possible that exposure of humans to cigarette smoke may compromise control of hypertension with guanethidine and other guanethidine-like drugs. We feel that the studies described will reveal significant information to indicate potential hazardous interactions between the antihypertensive, guanethidine, and the indirectly acting sympathomimetic substances, nicotine, and acetaldehyde.

9. Details of experimental design and procedures (append extra pages as necessary). The overall objective of the proposed research is to investigate the role of sympathetic innervation in the cardiovascular actions and interactions of the indirectly acting sympathomimetics, nicotine and acetaldehyde, present in cigarette smoke. In order to accomplish this objective, the investigation will be separated into two phases and will be carried out in small laboratory animals.

The first phase will consist of *in vivo* studies. Cardiovascular responses to intravenous administration of sympathomimetic agents will be examined in the anesthetized rat. This preparation is a convenient and economical model system in which blood pressure and cardiac changes can be monitored in whole animals and in preparations selectively altered by surgical and pharmacologic means. Observations made on the action of the sympathomimetic agents will be extended at the cellular level in isolated mammalian smooth muscle preparations in the second phase.

The second phase will consist of *in vitro* studies. Isolated smooth muscle preparations (perfused central ear artery and aortic strips from rabbits; isolated rat vas deferens) will be used in an attempt to compare the cellular actions and interactions of nicotine, acetaldehyde and tyramine. The ear artery more nearly reflects effects on arteries important in maintaining peripheral resistance; the aortic strips will be used for ^{14}C -norepinephrine kinetic studies; and the vas deferens preparation is an accessible smooth muscle which is densely innervated by the sympathetic nervous system. Sympathetic nerve function in the ear artery and vas deferens will be selectively altered by guanethidine, tetrodotoxin and calcium ion deprivation. Interactions between the sympathomimetic agents and ^{14}C -norepinephrine will be used to confirm the role of transmitter release in the cardiovascular actions of these agents.

In Vivo experiments: Male Wistar rats (250-300 g) are anesthetized with pentobarbital sodium (50 mg/kg) administered intraperitoneally. Body temperature will be maintained by means of an incandescent lamp. Mean arterial blood pressure is recorded from the right femoral artery through a cannula connected to a Statham pressure transducer. Intravenous injection of drugs is made into the system by means of a cannula inserted into the left femoral vein. A volume of 0.1 ml to 0.2 ml is used and washed in with 0.2 ml saline. An interval of 10 minutes will be used between doses of sympathomimetics; however, all parameters must have returned to preinjection control levels before subsequent injections are made. Heart rate is read directly with a tachograph (lead II) by means of fine needle electrodes inserted through the skin. Responses are recorded by means of a Grass polygraph. Bilateral adrenalectomy and vagotomy in the neck region will be carried out on anesthetized rats 30 minutes before injection of the first dose of drug. Rats treated with reserpine or guanethidine will be given 5 mg/kg by intraperitoneal injection for two days before the experiment.

1003542072

CURRICULUM VITAE

William LeRoy Heinrichs

Personal Data:

Birth Date
 Birth Place
 Citizenship
 Marital Status
 Children

REDACTED

REDACTED

Education:

High School	Central High School Collinsville, Oklahoma	1946-50
College: B.S.	Southwestern State College Weatherford, Oklahoma	1951-54
Medical School: M.D.	University of Oklahoma School of Medicine Oklahoma City, Oklahoma	1954-58
Rotating Intern	St. Anthony Hospital (Affiliate of University of Oklahoma Medical School) Oklahoma City, Oklahoma	1958-59
Ob-Gyn Resident	Harper Hospital (Affiliate of Wayne State University Medical School) Detroit, Michigan	1959-62
PHS Post-Residency Training Program: M. Sci. and Ph.D. - Biochemistry	Department of Obstetrics & Gynecology University of Oregon Medical School Portland, Oregon	1962-67

Academic Honors:

High School	Valedictorian of the graduating class	1950
College	Elected Alpha Phi Sigma and Beta Beta Beta (National Honor Fraternities)	1953

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qualitative differences between the acute and chronic administration of drugs, such as nicotine, which influence neuronal activity, it would seem of great importance to determine what differences in adrenergic nerve activity might exist between the acute administration of nicotine and following chronic exposure of this agent.

We have a very reproducible measure of the action of nicotine on adrenergic nerve activity that is: a) the release of ^3H -NE from the perfused guinea-pig heart and, b) the release of ^3H -NE from incubated brain slices. We also have quite a lot of experience in measuring the turnover of neurotransmitters (an in vivo marker for neuronal activity) as well as measurements of metabolic enzyme activity (monoamine oxidase and catechol-o-methyl transferase activity). The purpose of this present proposal therefore, is to study what influence the chronic administration of nicotine to rats and guinea-pigs has on several parameters of neuronal function such as: 1) the release of ^3H -NE from the perfused hearts by nicotine (model of peripheral adrenergic synapse). 2) the effect of nicotine on the release of labeled NE, dopamine and serotonin from brain slices obtained from discrete brain regions (model of central synapses) 3) the effect that the chronic exposure of nicotine has on the turnover of NE, dopamine and serotonin (in vivo marker of neuronal activity) and 4) the effect that the chronic exposure of nicotine has on adrenergic metabolic enzyme activity (MAO and COMT activity).

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These experiments will enable us to have a good comparison between the acute effect of nicotine on noradrenergic activity and the effect of nicotine on this activity after experimental animals have been exposed to the alkaloid for varying periods of time. This latter situation will more closely mimic what we might expect in

PHARMACOLOGY

Comm.

Dr. Gardner

Dr. Meier

Dr. Sommers

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., Inc.

110 EAST 59TH STREET

NEW YORK, N. Y. 10022

(212) 421-8885

Application for Research Grant

(Use extra pages as needed)

Date: 5/7/73

1. Principal Investigator (give title and degrees):

John A. Rosecrans, Ph.D.

Associate Professor

Dept. of Pharmacology

2. Institution & address:

Medical College of Virginia

Virginia Commonwealth University

Richmond, Virginia

3. Department(s) where research will be done or collaboration provided:

Department of Pharmacology

4. Short title of study:

State Dependent Properties of Nicotine Related Compounds

5. Proposed starting date:

1-1-74

6. Estimated time to complete:

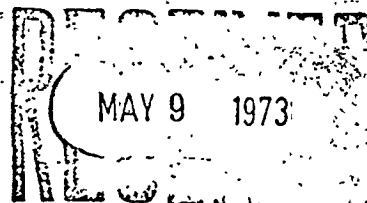
3 yrs

7. Brief description of specific research aims:

The major objective of this research will be to study the behavioral effects of various analogs and metabolites of nicotine, or compounds believed to have behavioral effects similar to nicotine. We hope to study these various compounds from the following points of view:

1. Specific behavioral effects of each compound to be studied.
2. The ability of such compounds to block nicotine's behavioral effect.
3. To test the ability of such drugs to act like nicotine, that is transfer to the nicotine state.

It is hoped that this study will allow us to establish the means by which we will be able to detect compounds suspected of having nicotine-like behavioral effects.



1003542104

TORU Tabei, M.D.

See publications for Heinrichs, W.L. for reprints for both Investigators.

1. Tabei, T. and Heinrichs, W.L. A study of the effect of the
2. Heinrichs, W.L. and Tabei, T. A study of the effect of the
3. Heinrichs, W.L. and Tabei, T. A study of the effect of the
4. Heinrichs, W.L. and Tabei, T. A study of the effect of the

2. Heinrichs, W.L. and Tabei, T. A study of the effect of the
3. Heinrichs, W.L. and Tabei, T. A study of the effect of the
4. Heinrichs, W.L. and Tabei, T. A study of the effect of the

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8C. Experience of Principal Investigator

The Principal Investigator has had a wide range of experience in studying the effects of drugs (particularly nicotine-type agents) and physiological manipulations on the synthesis, storage, release, uptake, and metabolism of catecholamines and closely related substances. Therefore, we feel we are well equipped to carry out studies involving the extraction, isolation, separation and measurement of labeled and unlabeled amines and metabolites. These techniques are, in fact, being carried out daily in the Principal Investigator's laboratory. In addition, we have had a lot of experience setting up and conducting studies on isolated and perfused tissue preparations. For these reasons, the Principal Investigator feels that he is well qualified for carrying out the experiments described in this research proposal.

9,10. Facilities Available and Additional Requirements.

These studies will be conducted in Dr. Westfall's laboratory which is housed in the Department of Pharmacology, Jordan Medical Education Building. These are new facilities which we moved into in April, 1972. Office and laboratory space consists of over 800 ft.². The primary laboratory is well equipped with glassware, ovens, water baths, stirrers, timing devices, etc. The following equipment is available: radiometric pH meter; Beckman pH meter; Packard Tri-Carb (3000 series) Scintillation Spectrometer; Farrand Model A photoelectric fluorometer with various filter combinations; Beckman Model B Spectrophotometer; two Mettler analytical balances; Facit Table Top Calculator; Polytron tissue homogenizers; three Harvard infusion pumps; Brush Mark II electronic recorder, Statham force and pressure transducers; perfusion and isolated tissue chambers; four metabolic and water baths; Technician autoanalyzer for catecholamine determinations.

In addition, other facilities are available which are shared with other Departmental members. These include: four cold rooms; a completely equipped enzyme preparation room; an effective working library with copying facilities; Aminco-Bowman spectrofluorometer; two other Technician autoanalyzers; four liquid Scintillation spectrometers; two Gilford 2400 spectrophotometers; automatic dishwasher, a range of refrigerated centrifuges including International PR-2, Sorvall RC-2, Beckman ultracentrifuges (L-2, L2-65B, L3-40) with various heads; Olivette Programma 101 computer; ReVCO ultraflow temperature freezer and two computer terminals.

Animals will be maintained under the care of full time veterinarians in the general animal quarters as well as a small animal room located on the same floor as the Department. This will be most convenient in maintaining adequate supervision of the administration of nicotine. The Medical School has a first rate library with over 1600 scientific journals currently on the subscription list.

1003542170

-12-

14. First year budget

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

John A. Rosecrans, Ph.D.

25

Technical

"Behavioral Technicians to be recruited"

100

REDACTED

Sub-Total for A

REDACTED

B. Consumable supplies (by major categories)

Research animals:

2000 rats
4000 mice

3000
1200

Sub-Total for B

4200

C. Other expenses (itemize)

Fringe Benefits - 10% of
Office supplies publication
Costs etc.
Travel to Scientific meetings

700
200
300

Sub-Total for C

1200

Running Total of A + B + C

REDACTED

D. Permanent equipment (itemize)

Additional Behavioral Equipment:
This will include two operant chambers and
an automated one way-shuttle box (Leigh Valley
Electronics, Inc. and Lafayette Instrument Co.)

Sub-Total for D

5000

E

1860

Total request

20260

15. Estimated future requirements

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip.	Indirect Costs	Total
Year 2	REDACTED	4200	940		1831	
Year 3	REDACTED	4200	980		1947	

REDACTED

1003542115

13. "The Clinical Epidemiology of Sporadic Measles in a Highly Immunized Population," W. Schaffner, A.E. Schluederberg, and E. B. Byrne, *New Eng. J. Med.*, 279, 783 (1968).
14. "Rubella Antibodies in Rhode Island Women of Child-bearing Age," E. B. Byrne, R. L. Petrelli, W. Schaffner, and M. C. Hinchliffe, *Pub. Health Rep.*, 84, 139 (1969).
15. "A Smallpox Vaccination Campaign for Hospital Personnel in Rhode Island," W. Schaffner and R. M. Adair, *Pub. Health Rep.*, 84, 425 (1969).
16. "Hospital Outbreak with Group-A Streptococci Traced to an Asymptomatic Anal Carrier," W. Schaffner, L. B. Lefkowitz, J. S. Goodman, and M. G. Koenig, *New Eng. J. Med.*, 280, 1224 (1969).
17. "Botulism," Chapter 26, Vol. III of *Tice's Practice of Medicine*, Hoeber Medical Division, Harper and Row Publishers, Inc., Hagerstown, Md., 1970 (W. Schaffner and M. G. Koenig).
18. "Infant Immunization Surveillance: Cost Versus Effect. A Prospective Controlled Evaluation of a Large Scale Program in Rhode Island," E. B. Byrne, W. Schaffner, E. Dini, and G. W. Case, *J. A. M. A.*, 212, 770 (1970).
19. "Two Syndromes Following Rubella Immunization. Clinical Observations and Epidemiological Studies," A. W. Kilroy, W. Schaffner, W. F. Fleet, L. B. Lefkowitz, D. T. Karzon, and G. M. Fenichel, *J. A. M. A.*, 214, 2287 (1970).
20. "Severe Influenza Virus Pneumonia in the Pandemic of 1968-1969," R. F. Burk, W. Schaffner, and M. G. Koenig, *Arch. Int. Med.*, 127, 1122 (1971).
21. "Superinfection in Lymphoreticular Diseases." *Annual Review of Medicine*, Vol. 22. Annual Reviews, Inc., Palo Alto, Calif., 1971, pp 25-38 (Z. A. McGee, W. Schaffner, and M. G. Koenig).
22. "Innovation in Communicable Disease Reporting," W. Schaffner, H. D. Scott, B. J. Rosenstein, and E. B. Byrne, *HSMHA Health Rep.*, 86, 431 (1971).
23. "The Use of Marginal-punched Data Cards in Surveillance of Hospital-acquired Infection," L. B. Lefkowitz, G. B. Lavelly, and W. Schaffner, *HSMHA Health Rep.*, 86, 953 (1971).
24. "Measles Eradication: The Impossible Dream?" W. Schaffner, *Proceedings of the Eighth Immunization Conference*, Center for Disease Control, USPHS, HSHMA, DHEW, Atlanta, Ga., pp 15-16, 1971.
25. "Efficacy and Safety of Topical Lysostaphin Treatment of Persistent Nasal Carriage of *S. aureus*," K. E. Quickel, R. Selden, J. R. Caldwell, N. S. Nora, and W. Schaffner, *Appl. Micro.*, 22, 446 (1971).

1003542198

BUDGET:
 CHARACTERISTICS OF SMOKERS VS NON-SMOKERS RENEWAL
 FEBRUARY 1, 1974-JANUARY 31, 1975

A. Salaries (Personnel by names or category)	% time	Amount
Professional		
Gary D. Friedman, M.D., M.S.	20%	\$ 8,270
Carl C. Seltzer, Ph.D.	20%	5,300
A. B. Siegelau, M.S.	30%	7,700
Loring G. Dales, M.D.	20%	5,330
Technical		
Programmers (2)	200%	27,640
Clerk-Typist	75%	8,830
Sub-Total		\$63,070
B. Consumable Supplies (list by categories)		
Office supplies & copying		1,100
Sub-Total		\$1,100
C. Other Expenses (itemize)		
Travel by Dr. Seltzer, Boston-Oakland (3 trips each year)		1,800
Travel to scientific meetings (4 trips)		2,000
Data processing		18,000
Key punching		500
Sub-Total		\$22,300
D. Permanent Equipment (itemize)		
		0
Sub-Total		0
E. Overhead (15% of A+B+C)		\$12,970
Total		\$99,440

*ok
207*

1003542216

49. "A Spot Test for Detection of Lead in Paint," J. W. Sayre and D. J. Wilson, *Pediatrics*, 46, 783 (1970).
50. "Quantum Vibrational Transition Probabilities in Atom-Diatomic Molecule Collisions. V. Effects of Mass and Well Depth," D. J. Wilson, *J. Chem. Phys.*, 54, 540 (1971).
51. "Quantum Vibrational Transition Probabilities in Atom-Diatomic Molecule Collisions. A Tractable Three-Dimensional Model," D. J. Wilson and D. J. Locker, *J. Chem. Phys.*, 57, 5393 (1972).
52. "DDT Concentrations in Human Milk," D. J. Wilson, D. J. Locker, C. A. Ritzen, and J. T. Watson, *Amer. J. Diseases Children*, 125, 814 (1973).
53. "Effect of Nonequilibrium in Gas Chromatography," J. P. Muth, D. J. Wilson, and K. A. Overholser, submitted to *J. Chromatography*.
54. "Hexachlorophene Levels in Human Milk," Robert West and David J. Wilson, manuscript in preparation.
55. "Lead Levels in Human Milk," H. Kenneth Dillon and David J. Wilson, manuscript in preparation.
56. "Non-Ideal Line Shapes in Gas Chromatography," Sheng-Da Huang, John W. Wilson, and David J. Wilson, manuscript in preparation.

1003542194

Central and Peripheral Mechanism of action of
nicotine; Neuropharmacology; Autonomic Pharmacology

RESEARCH AND PROFESSIONAL EXPERIENCE:

- 1972- Director, Medical School Pharmacology Course
- 1969- Associate Professor of Pharmacology, Univ. of Virginia School of Medicine
- 1969- Chairman, Committee on Graduate Studies and Department Graduate Advisor
- 1965-69 Assistant Professor of Pharmacology, University of Virginia School of Medicine
- 1964-65 Assistant Professor of Pharmacology, West Virginia University Medical Center
- 1963-64 Postdoctoral Fellow of National Heart Institute, Department of Physiology, Karolinska Institute, Stockholm, Sweden (Professor U.S. von Euler, Advisor)
- 1962-63 Instructor in Pharmacology, West Virginia University Medical Center

12. PRINCIPAL PUBLICATIONS DURING THE PAST SEVEN YEARS:

1. Westfall, T. C. Tobacco alkaloids and the release of catecholamines in Tobacco Alkaloids and Related Compounds, Ed. by U.S.von Euler, Pergamon Press, 4: 179, 1965.
2. Westfall, T. C. Effect of nicotine and nicotine analogues on tissue and urinary catecholamines in the rat. Acta Physiol. Scand., 63: 77, 1965.
3. Westfall, T. C. Uptake and exchange of catecholamines in rat tissues after d-and 10 adrenaline. Acta Physiol. Scand., 63: 336, 1965.
4. Westfall, T. C. and Peach, M. J. Action of angiotensin on myocardial and renal catecholamines in the rabbit. Biochem. Pharmacol., 14: 1916, 1965.
5. Westfall, T. C., Cippoloni, B. and Edmundowicz, A. Influence of propranolol on the hemodynamic changes and plasma catecholamine levels following cigarette smoking and nicotine. Proc. Soc. Exp. Biol. Med., 123: 174, 1966.

1003542172

FROM WALTER B. ESSMAN, M.D., Ph.D.
Re Grant Application #467C
May 21, 1973

RESEARCH PROPOSAL EVALUATION
Dr. Thomas C. Westfall

Action of Nicotine on Peripheral and
Central Neurons In Animals Chronically Exposed to Nicotine

The proposal is a well-organized, carefully conceived series of studies which have been developed logically from previous experiments originating in the investigator's laboratory. It would seem that many of the techniques proposed for use in the investigation have been carefully developed and well utilized. The proposed use, particularly of regional tissue from the central nervous system, is interesting and the use of the perfused heart preparation is also most appropriate. I believe that the proposal generally carefully considered some of the important aspects of nicotine action and are quite sound both methodologically and theoretically. One point made in the proposal that I would take some exception to is the ready willingness of the investigator to correlate the effects of nicotine in these proposed experiments with the effects of tobacco smoking in man. I doubt that this could or even should be considered, but I do not think that it detracts appreciably from the quality of the proposed experiments or their significance. I believe that the proposal in general is quite good and that the investigator has presented an impressive array of experiments which will yeild reasonable results.

1003542182

Academic Honors (Cont'd):

Medical School	Student Research Achievement Award in Biochemistry	1958
Ob-Gyn Residency	Second Award for a Scientific Paper	1962
Post-Residency Training	Appointed Macy Fellow in Obstetrics and Reproduction	1966
Career	Presidents First Award for Research, American College of Obstetricians and Gynecologists	1970

Academic Appointments:

Clinical Instructor	Department of Obstetrics & Gynecology University of Oregon Medical School Portland, Oregon	1965-67
Assistant Professor	Department of Obstetrics and Gynecology University of Washington School of Medicine Seattle, Washington	1967-69
Associate Professor	Department of Obstetrics and Gynecology University of Washington School of Medicine Seattle, Washington	1969-72
Professor	Department of Obstetrics and Gynecology University of Washington School of Medicine Seattle, Washington	1972-

Hospital Appointments:

Attending Staff	Good Samaritan Hospital Portland, Oregon	1965-67
Attending Staff	University Hospital, and Harborview Medical Center, Seattle, Washington	1967-
Consultant	U.S. Public Health Service Hospital Seattle, Washington	1971-
Consultant	Madigan General Hospital Tacoma, Washington	1971-

1003542231



COMMONWEALTH OF PENNSYLVANIA
DEPARTMENT OF PUBLIC WELFARE

EASTERN PENNSYLVANIA PSYCHIATRIC INSTITUTE
Henry Avenue and Abbottsford Road
Philadelphia, Pennsylvania 19129

TELEPHONE
AREA CODE 215, 848-6000

June 6, 1973

Dr. Fredeirck W. Norsiek
Associate Scientific Director
The Council for Tobacco Research-USA, Inc.
10 East 59th Street
New York, New York 10022

SUBJECT: Review of Grant Application #909

Dear Dr. Norsiek:

It is my pleasure to be able to assist an independent research sponsoring agency such as the Council for Tobacco Research.

I have read the grant application entitled "State Dependent Properties of Nicotine Compounds" submitted by John A. Rosecrans and believe that I am competent to scientifically evaluate the work described therein. I recommend that this grant only be funded at a reduced level, with the investigator specifically requested to use the approved funding only for experiments of the type described on pages 7-10 of his application. My reasons for making this recommendation are outlined in the following paragraphs.

State dependent learning, and the resulting ability of drugs to control differential responding, are very interesting drug effects which are produced by most "self-administered" drugs including nicotine. Drug discrimination experiments of the type proposed by Rosecrans on pages 7-10 of his application have a demonstrated utility in comparing and categorizing the CNS effects of drugs. Rosecrans, himself, has recently done some very significant work in this area investigating the effects of depletion of various biogenic amines on drug discrimination. The use of drug discriminations as a tool for comparing the CNS effects of various drugs has certain distinct advantages over most other behavioral techniques. I think that work in the general area of this application is well worth supporting and falls within the area of interest of the Tobacco Research Council, in so far as I understand it's goals.

The drug discrimination studies proposed on pages 7-10 of this application will almost certainly yield useful data regarding the relationships between nicotine-like drugs. The PI has previously differentiated between peripherally and centrally acting nicotine-like agents using these procedures. He has also reported a significant difference between the discriminable effects of nicotine and lobeline. Although there are some small areas in the proposed research where difficulties may be encountered, the research plan appears basically sound.

1003542123

ABSTRACTS

1. Tabei, T., and Troen, P.: Studies of C-6 hydroxylation of C₂₁ steroids in human placenta. *J. Clin. Invest.* **48**: 82a, 1969. (Presented at the 61st Annual Meeting of the American Society for Clinical Investigation, Atlantic City, 1969).
2. Tabei, T., and Heinrichs, W.L.: Puberty and hepatic 16-oxygenation of 3 β -hydroxyandrost-5-en-17-one (DHA). The 53rd Annual Meeting of The Endocrine Society, San Francisco, 1971.
3. Heinrichs, W.L., Haga, H., and Tabei, T.: Progesterone 5 α -reductase activity in cell-free homogenates of rat brain and other tissues. The 19th Annual Meeting of The Society for Gynecologic Investigation, San Francisco, 1972, No. 75.
4. Tabei, T., Haga, H., and Heinrichs, W.L., and Hermann, W.L.: Metabolism of progesterone by the brain and the pituitary gland. *Clin. Res.* **21**: 206, 1973.

1003542243

Page 3b.

Item 10. continued

Fetoplacental Enzymology (Obstetrics and Gynecology). A Biochemical Endocrinology Laboratory in the Department of Obstetrics and Gynecology comprises 400 square feet of working space, including a hood, Barbara Coleman gas liquid chromatograph, evaporative extractors and temperature-related water bath where Dr. Toru Tabei will carry out assays. He will have the use of the liquid scintillation spectrometers in the adjacent instrument room of the department. In addition, an 80-foot-square cold room with a Spiuco L-2 preparative ultra centrifuge is available for his use.

1003542227

ARTICLES

IN PRESS AND IN PREPARATION

1. Tabei, T., and Heinrichs, W.L.: Enzymatic oxidation and reduction of C₁₉- Δ^5 -3 β -hydroxysteroids by hepatic microsomes. III. 7-oxygenation of 3 β -hydroxyandrost-5-en-17-one (DHA) during puberty in rats. Submitted to Endocrinology, 1973.
2. Tabei, T., and Heinrichs, W.L.: Enzymatic oxidation and reduction of C₁₉- Δ^5 -3 β -hydroxysteroids by hepatic microsomes. IV. Critical period for the neonatal differentiation of certain mixed-function oxidases. Submitted to Endocrinology, 1973.
3. Haga, H., Tabei, T., and Heinrichs, W.L.: Progesterone 5 α -reductase activity in cell-free homogenates of rat brain and other tissues. In preparation, 1973.
4. Tabei, T., and Heinrichs, W.L.: Progesterone 5 α - and 20 α -reduction by rat brain and pituitary gland. In preparation, 1973.

1003542244

MANUSCRIPTS IN PRESS

John T. Conrad, Ph.D.

1. Conrad, J.T. and Onwudiwe, F.: The effects of the prostaglandins, PGE and PGF_{2d} upon the in vitro isthmus, ampulla and uterus of the estrus rabbit. Prostaglandins (July, 1973).
2. Conrad, J.T.: "Uterine biomechanics" in Biomechanics, ed., D.N. Ghista. Mosby Co., St. Louis, Mo.

1003542250

Turnover of Norepinephrine.

At various periods of time of nicotine treatment, measurements of norepinephrine turnover will be made. Animals will be injected with alpha-methyl tyrosine methylester (α MPT) i.v. in a dose of 200 mg/kg followed by a subsequent dose of 100 mg/kg 2 hours later. Animals will be killed at 2, 4, 6 and 8 hours after α MPT and heart and brain removed for extraction and analysis of NE. The tissues will be dissected, washed in saline, weighed and homogenized in 5% trichloroacetic acid (heart) or 0.4 N perchloric acid (brain) by an Ultra-Turrax homogenizer. After centrifugation and absorption of the catecholamines on alumina columns, NE will be measured using the automated trihydroxyindole procedure (23). Analysis will be made on the whole heart and discrete brain regions including medulla-pons, striatum, hypothalamus, cerebellum, cortex, and brain stem. These regions will be dissected out according to the procedure described above. Turnover rates will be calculated by multiplying the steady-state level of NE by the fractional rate constant for the decline in endogenous NE after α MPT (27-29)

Monoamine Oxidase Activity.

MAO activity will be carried out according to the method of Wurtman and Axelrod (30). At various periods of time of nicotine administration, animals will be killed by decapitation and 200 mg of liver and 1 whole heart will be taken and homogenized in cold isotonic KCl. The tissue will be homogenized so that the final tissue concentration of liver will be 2 mg/ml. Tissue homogenates will then be incubated in a reaction mixture containing 0.1 M phosphate buffer and

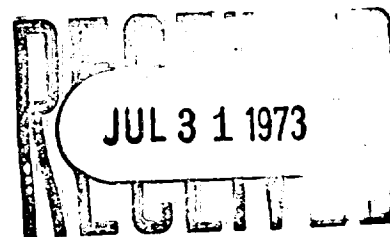
1003542165

CONFIDENTIAL

Nov. 16, 1972 -

July 20, 1973

Gary D. Friedman, M.D.
Department of Medical Methods Research
Kaiser Foundation Research Institute
3779 Piedmont Avenue
Oakland, California 94611



Overview

Beginning in February, 1971 and made possible by a grant from the Council for Tobacco Research-U.S.A., we undertook a large scale epidemiologic study of the Characteristics of Smokers and Non-Smokers using Kaiser-Permanente multiphasic examination data collected on 111,000 subjects during the years 1964-1968. Since that time we have generated literally volumes of computer output with a great deal of valuable information. These basic data analyses are now continuing at a slower rate as we have gradually shifted our emphasis toward secondary analyses aimed at completing the picture outlined by the basic analyses. That is, the basic analyses have revealed a number of interesting and important findings but these raise questions which must be pursued further if we are to publish papers that meet high scientific standards. For example, the finding of a lower serum albumin concentration in smokers than in non-smokers led us to determine whether smoker-nonsmoker differences in alcohol consumption or in the prevalence of liver disease could account for the differences in albumin levels, which they did not.

Underlying this effort to "complete the picture" is our desire to publish our findings and present them to the scientific community. A number of papers have reached various stages of completion and these are summarized below. In addition to the papers which directly satisfy the project goals, three papers have resulted which are wholly or in part byproducts of the data collection and analysis that we have carried out with Council of Tobacco Research-U.S.A. support. These are also mentioned.

Papers Directly Satisfying Project Goals

Published Papers

1. Friedman, G.D., Seltzer, C.C., Siegelau, A.B., Feldman, R., and Collen, M.F.: Smoking among white, black and yellow, men and women: Kaiser-Permanente Multiphasic Health Examination Data, 1964-1968. Amer. J. Epidemiol. 96:23-35, 1972.
2. Friedman, G.D., Siegelau, A.B., Seltzer, C.C., Feldman, R. and Collen, M.F.: Smoking habits and the leukocyte count. Archives of Environmental Health. 26:137-143, 1973.

(Presented to the Society for Epidemiological Research, Houston, May, 1972)

Papers Accepted For Publication

3. Seltzer, C.C., Friedman, G.D., Siegelau, A.B.: Smoking and drug consumption in white, black and oriental men and women. To be published in the American Journal of Public Health.

1003542212

Academic Appointments (Cont'd):

Assistant Professor	Department of Obstetrics & Gynecology University of Tokyo Tokyo, Japan	1969-70
Research Assistant Professor	Department of Obstetrics & Gynecology University of Washington School of Medicine Seattle, Washington	1971-

Hospital Appointments:

Clinical Attending Staff	Department of Obstetrics & Gynecology University Hospital University of Tokyo Tokyo, Japan	1968-70
Clinical Attending Staff	Sanraku Hospital (Affiliate of the University of Tokyo Tokyo, Japan	1968-70

1003542241

Item 9, continued

profound changes in certain oxidizing enzymes of the liver, and the presence of peroxidase and cholesterol side-chain cleavage enzymes of the adrenal cortex. In this project, representative portions of the organs will be fixed, sectioned and stained for histologic examination.

Quantitation of Plasma Lipids and Hormones

The following list will be determined by the Department of Laboratory Medicine:

A. Total cholesterol assays

Method: spectrophotometric analysis

- References: 1. Zlatos A., Zak B. and Boyle A. J. Lab. & Clin. Med., Vol. 41, p. 486, 1953.
2. Zak B., Dickerman R.C., White E.G. and Cherney P.J. Am. J. Clin. Pathology, Vol. 24, p. 1307, 1954.
3. Leffler H. Am. J. Clin. Pathology, Vol. 31, p. 310, 1959.

B. Phospholipid assays

Method: spectrophotometric analysis after perchloric acid digestion

- References: 1. Zilversmit D.B. and Davis A.K. J. Lab. & Clin. Med., Vol. 35, p. 155, 1950.
2. Bantlett G.R. J. Biol. Chemistry, Vol. 234, p. 466, 1959.

C. Triglyceride assays

Method: fluorimetric analysis

- References: 1. Noble R.P. and Campbell F.M. Clin. Chem., Vol. 16, p. 166, 1970.
2. Kessler G. and Lederer H. "Fluorimetric Measurement of Triglycerides," Automated Analytical Chemistry; Technicon Symposia (series). ed., L.T. Speggs. New York: Mediad, Inc., p. 341, 1965.

D. Progesterone assays

Method: competitive protein binding

- References: 1. Niell J.D., Johansson E.D., Datta J.K. and Knobil E. J. Clin. Endocr., Vol. 27, p. 1167, 1967.
2. Stone S., Nakamura R.M., Mishell D.R., Jr. and Thorneycroft I.H. Steroids, Vol. 17, p. 411, 1971.
3. Schiller H., Conrad S., Mahler E., Cox D. and Heinrichs W.L. (in preparation)

E. Estradiol assays

Method: radioimmunoassay analysis

1003542222

10. Protocol for the ensuing year:1. Action of Nicotine on the Medulla:

A. Measurement of cyclic AMP levels. Since our initial experiments indicate that nicotine augments the synthesis and release of adrenal cyclic AMP (see Summary Progress Report), the next step will be to attempt to correlate cyclic AMP levels with catecholamine release, in order to ascertain whether the observed increases in cyclic AMP are directly responsible for the nicotine-induced enhanced rate of secretion.

Thus, cat adrenal glands will be perfused in situ with Locke's solution plus various concentrations of nicotine or acetylcholine for varying time intervals, the perfusate collected from a polyethylene cannula in the adrenolumbar vein and assayed for catecholamines by fluorometry (Rubin and Jaanus, 1966) and cyclic AMP by radioimmunoassay (Steiner et al., 1969). The adrenals will also be analyzed for cyclic AMP. It will be of interest to observe whether the stimulant effects of nicotine on catecholamine release can be quantitatively and temporally correlated with the synthesis and release of cyclic AMP.

An indication of the cellular localization of medullary cyclic AMP could be obtained by differential centrifugation techniques after homogenizing the medulla in isotonic sucrose. Although manipulative procedures alter cyclic AMP concentrations, it still may be possible to ascertain in which cell fraction (mitochondrial, microsomal, granular) the cyclic nucleotide is mainly localized, by analysis of each fraction. Such data may aid in elucidating the role of cyclic AMP in the secretory mechanism and its possible relation to the action of calcium.

B. Effect of cyclic nucleotide and phosphodiesterase inhibitors. If tissue concentrations of cyclic AMP directly modulate the rate of nicotine-mediated catecholamine release, then perfusion with cyclic nucleotide or inhibitors of phosphodiesterase (the enzyme responsible for cyclic AMP degradation), such as theophylline, might be expected to mimic the effects of nicotine. Therefore, additional experiments will be carried out to discern whether cat adrenal glands perfused with dibutyryl cyclic AMP and/or theophylline increase spontaneous catecholamine release or potentiate the secretory response to nicotine and to acetylcholine.

Previous studies have shown that the action of nicotine, acetylcholine and other medullary secretagogues is associated with an increase in the permeability of the chromaffin cell membrane, with a subsequent increase in transmembrane calcium flux (Rubin, 1970). The experiments which are planned for the ensuing year may provide valuable information on the role of cyclic AMP in the molecular events associated with the calcium-dependent activation of the secretory mechanism.

2. Action of Nicotine on the Cortex:

A. Effect of exogenous cyclic nucleotide and inhibitors of phosphodiesterase. Since our previous investigations have demonstrated that nicotine potentiates the steroidogenic activity of ACTH in isolated cat cortical cells,

1003542132

4.

14. First year budget:

A. Salaries (give names or state "to be recruited")

Professional (give % time of investigator(s)
even if no salary requested)

% time Benefits Amount

	Position	% time	Benefits	Amount
W. LeRoy Heinrichs	(Principle Investig.)	10		\$
Toru Tabei	Res. Ass't. Prof. (Ob/GYN)	50		REDACTED
Pearl Namkung	Res. Tech. II (Ob/GYN)	100		
To be recruited	Res. Tech. I (Pharmacol.)	100		
To be recruited	Secretary (Ob/GYN)	50		REDACTED
Alan Fantel	Ass't. Teratologist (Peds.)	50		
John Conrad (Ob/GYN)	Co Investigator	10		REDACTED
Mont Juchau (Pharmacology)	"	15		
Felix Freund (Anesthesiology)	"	10		
Harvey Schiller (Ob/GYN)	"	10		
Thomas Shepard (Pediatrics)	"	5		

Sub-Total for A REDACTED

B. Consumable supplies (by major categories)

Obstetrics and Gynecology

Toru Tabei	3,000
John Conrad	1,000

Pharmacology

Mont Juchau	3,000
-------------	-------

Sub-Total for B 7,000

C. Other expenses (itemize)

Publication costs	750
Equipment	600
Laboratory Medicine (plasma, lipids, hormones)	4,000
Patient Costs (30 patients at \$100 each)	3,000
Travel (3 cross country trips)	1,800

Sub-Total for C 10,150Running Total of A + B + C REDACTED

D. Permanent equipment (itemize)

Sub-Total for D

E. Indirect costs (15% of A+B+C) (minus equipment)

E 9,458Total request REDACTED

15. Estimated future requirements.

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2						
Year 3						

1003542228

ok
2/17

Other Appropriate Information:

1965- Consultant to Anesthesiology Department, Group Health
 Hospital, Seattle, Washington

12/6/71:tc

1003542267

27. Freund, F.G. and Merati, J.K.: Errors in assessing neuromuscular blockade. Anesthesiology (in press).

1003542270

7/18/73:tc

MANUSCRIPTS
IN PREPARATION

1. Cox, D., Heinrichs, W.L., Paulsen, C.A., Conrad S., Schiller, H.S., Henzl, M., and Herrmann, W.L.: Perturbations of the Human Menstrual Cycle by Oxymetholone (1973).
2. Schiller, H.S., Conrad, S., Cox, D., Heinrichs, W.L., and Herrmann, W.L.: Plasma Progesterone by Competitive Protein Binding Assay: A Comparison of Two Methods and Evaluation as an Indication of Ovulation (1973).

1003542264

IN PRESS

1. Heinrichs, W.L.: Steroid hydroxylases and drug-metabolizing enzymes in hepatic microsomes. I. Pregnancy and administration of phenobarbital or etiocholanolone. Submitted and being revised for *Archiv. Biochem. Biophys.*, 1973.
2. Tabei, T., and Heinrichs, W.L.: Enzymatic oxidation and reduction of C_{19} - Δ^5 - 3β -hydroxysteroids by hepatic microsomes. III. 7-oxygenation of 3β -hydroxyandrost-5-en-17-one (DHA) during puberty in rats. Submitted to *Endocrinology*, 1973.
3. Tabei, T., and Heinrichs, W.L.: Enzymatic oxidation and reduction of C_{19} - Δ^5 - 3β -hydroxysteroids by hepatic microsomes. IV. Critical period for the neonatal differentiation of certain mixed-function oxidases. Submitted to *Endocrinology*, 1973.

IN PREPARATION

1. Haga, H., Tabei, T., and Heinrichs, W.L.: Progesterone 5α -reductase activity in cell-free homogenates of rat brain and other tissues.
2. Tabei, T., and Heinrichs, W.L.: Progesterone 5α - and 20α -reduction by rat brain and pituitary gland.
3. Forster, M.S., and Heinrichs, W.L.: In vitro binding of DDT and its homologues to estrogen receptors in target tissues of rats.
4. Forster, M.S., and Heinrichs, W.L.: Estrogen receptor binding capacity in hyperplastic and neoplastic human endometrium.
5. Forster, M.S., Wyss, H., Gellert, R.J., and Heinrichs, W.L.: Changes in estrogen receptor in target tissues after neonatal estrogen-induced constant estrus.

1003542239

16. Other sources of financial support: (for M. R. Juchau, Ph.D.)

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Biotransformation of Drug Substrates in Human Fetal Tissues	National Foundation CRBS 250	15,975	July 1, 1973 - June 30, 1974
Metabolism of Drug Substrates by Human Placenta	NIH - HD 04839	24,980	January 1, 1973 - December 31, 1973

PENDING OR PLANNED

Title of Project	Source (give grant numbers)	Amount	Inclusive Dates

1003542229

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made."

Principal investigator

Typed Name W. LeRoy Heinrichs, M.D., Ph.D.Signature *W. LeRoy Heinrichs* Date 7-23-73Telephone (206) 543-3580

Area Code Number Extension

Checks payable to

Donald R. Baldwin, Director
Office of Grants and Contract Services

Mailing address for checks

211 Administration Bldg. AG-50University of WashingtonSeattle WA 98195

Responsible officer of institution

George W. Farwell
George W. Farwell, Ph.D.
Vice President for Research

Telephone (206) 543-0151

Area Code Number Extension

- References: 1. Abraham G.E. J. Clin. Endocr., Vol. 29, p. 866, 1969.
2. Abraham G.E. Biochem. Med., Vol. 3, p. 365, 1970.

F. Carboxyhemoglobin assays

Method: spectrophotometric

- Reference: 1. Maas A.H.J., Hamelink M.L. and DeLeeuw R.J.M.
Clin. Chim. Acta, Vol. 29, p. 303, 1970.

G. Human Chorionic Somatomammotrophin (HCS) assays

Method: radioimmunoassay

- Reference: 1. Spellacy W.N., Carlson K.L. and Birk S.A. Am. J.
Ob. Gyn., Vol. 96, p. 1164, 1966.

H. Human Chorionic Gonadotrophin (HCG) assays

Method: radioimmunoassay

- Reference: 1. Paulsen C.A., Gordon D.L., Carpenter R.W., Gandy
H.M. and Drucker W.D. Recent Prog. Horm. Res.,
Vol. 24, p. 321, 1968.

Physiological Studies In Vitro on Umbilical Cord

Human umbilical arteries will be obtained as soon as possible after delivery. Up to five segments will be removed from the sample and placed in chambers and connected to sensitive isometric tension transducers. Initially a hypoxic gas mixture containing 8% O₂, 5% CO₂ and 87% N₂ will be bubbled into the chamber to induce a relaxation of the muscular segment (Bor I. and Buntheroth W.G. "In Vitro Response to Oxygen of Human Umbilical Arteries and of Animal Ductus Arteriosus," Canadian J. Physiol. & Pharm., Vol. 48, pp. 500-502, 1970.). After a period of relaxation (approximately one to two hours) a hyperoxic gas mixture of 95% O₂ will be introduced for a period of 30 minutes. This technique usually induces a contraction in the arterial segments. Gas mixtures may be altered several times to insure a series of contractions and relaxations.

After an initial contraction-relaxation cycle, various amounts of nicotine or prostaglandins (the exact amounts to be determined by experimentation) will be added to the bath and an additional contraction-relaxation cycle produced. The various levels in sensitivity in the groups of umbilical artery segments will be noted then and compared. It will be feasible by this method to run an entire dose-response curve for a particular patient by using a multiple bath arrangement.

Fetoplacental Enzymology (Pharmacology)

Aryl hydrocarbon hydroxylase activities will be assayed by measuring the appearance of the fluorescent hydroxylated metabolites according to modifications of the method of

1003542223

Comm.

Dr. Bing
Dr. Loosli
Dr. Sommers

MISCELLANEOUS

#1932

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022
(212) 421-8985

JUL 30 1973

Application for Research Grant

Date: 7-20-73

(Use extra pages as needed)

1. Principal Investigator (give title and degrees):

W. LeRoy Heinrichs, M.D., Ph.D.
Professor, Department of Obstetrics and Gynecology
Director of Endocrine Research

2. Institution & address:

University of Washington
Seattle, Washington 98195

3. Department(s) where research will be done or collaboration provided:

Obstetrics and Gynecology, University of Washington
Pediatrics, University of Washington
Pharmacology, University of Washington
Anesthesiology, Harborview Medical Center, Seattle, Washington

4. Short title of study:

The Effect of Smoking on Adaptive Changes of Previaible Human Pregnancies

5. Proposed starting date: January 1, 1974

6. Estimated time to complete: one year

7. Brief description of specific research aims:

The cardiopulmonary and metabolic-endocrine adaptations in the placental and fetal morphology and enzymology of women seeking termination of 14-16 week pregnancies will be compared in nonsmokers and in women smoking cigarettes or marijuana.

- A. Cardiopulmonary studies; the cardiac output, peripheral resistance, arterial blood gases, tidal volume and minute volume will be determined one day preabortion and six weeks postabortion.
- B. Metabolic-endocrine; blood lipid assays will include total cholesterol and esters, phospholipids and triglycerides. Plasma hormones will be HCG (human chorionic gonadotrophin), HCS (human ^{chorionic}somatotrophin), progesterone, and estradiol. In addition to routine preoperative assays, plasma carboxyhemoglobin concentrations will be ascertained.
- C. Fetal morphology; the total body measurements, fetal organ and placental weights and histopathology of the organs will be determined.
- D. Contractile behavior *in vitro* of umbilical arteries and their sensitivities to nicotine and prostoglandin will be determined.
(see attached)

1003542219

CURRICULUM VITAE

John T. Conrad, Ph.D.

Personal Data

Date of Birth
Place of Birth
Citizenship
Marital Status
Children

REDACTEDEducation

B.A.	New York University Washington Square College New York, New York	1951
M.S.	New York University New York, New York	1955
Ph.D.	New York University New York, New York	1961

Academic Appointments

Research Assistant	Department of Biology Washington Square College New York University New York, New York	1952-53
Research Assistant	Sloan-Kettering Institute for Cancer Research	1953-54
Teaching Fellow	Department of Biology Washington Square College New York University New York, New York	1955-57
Research Assistant	Department of Internal Medicine Neurology Section Yale University School of Medicine New Haven, Connecticut	1957-60
Instructor	Department of Physiology Yale University School of Medicine New Haven, Connecticut	1960-62

1003542246

- E. Placental enzymes (aryl hydrocarbon hydroxylase, cholesterol sidechain-splitting enzyme, and androgen aromatase activities will be quantitated in vitro).
- F. Enzyme activities in fetal liver, adrenal gland, kidney, intestine and brain will be determined selectively (see below):

<u>Organ</u>	<u>Enzyme Activity</u>
Placenta (Pharmacology)	aryl hydrocarbon hydroxylase cholesterol sidechain-splitting enzyme androgen aromatization
Liver (Obstetrics and Pharmacology)	DHA 7 α - hydroxylase 7 β - hydroxylase 16 α - hydroxylase 7 - hydroxysteroid dehydrogenase 16 α - hydroxysteroid dehydrogenase 17 β -hydroxysteroid dehydrogenase w-oxidation fatty acids aryl hydrocarbon hydroxylase azo dye N-demethylase
Adrenal (Pharmacology)	aryl hydrocarbon hydroxylase
Brain (Obstetrics)	progesterone 5 α reductase progesterone 20 α hydroxysteroid dehydrogenase
Intestine (Pharmacology)	glucuronyl transferase
Kidney (Pharmacology)	azo dye N-demethylase naphthylamine N-hydroxylase
Plasma (Pharmacology)	procaine esterase
Umbilical Cord (Obstetrics)	contractility studies with nicotine, prostaglandin, adrenergic and cholinergic stimulation

1003542220

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

August 3, 1973

Grant application #787B

EPIDEMIOLOGY

To: The committee comprising Drs. Gardner, Jacobson, Loosli and Sommers

Subject: Gary D. Friedman, M.D., Kaiser Foundation Research Institute
Continuation Application #787B (no commitment)
"Characteristics of Smokers and Non-Smokers"

History

Grant #787, with renewals and continuations, has supported this study since 1971.

The current grant, awarded without assurance of continued support provides an annual level of approximately \$100,000. The award letter stated that a renewal application, with a progress report, would receive consideration.

Application #787B requests \$99,440. for the ensuing year.

Documents Submitted

Attached is application dated July 27, 1973 incorporating Progress Report #3, November 16, 1972 - July 20, 1973. (An incorrect application form has been used as this request competes without commitment.)

Comment

The progress report lists a rather impressive number of papers, either published or else in various stages of submittal or preparation. In our files are drafts, manuscripts, or reprints of all of these. Copies of any will be sent to you on request. Dr. Hockett may decide to send you a selection.

FWN:gh

FWN
F.W.N.

Attachments

1003542209

#912-McCLUGAGE

1003542275

13. Recent and pertinent publications (reprints attached):

1. M.R. Juchau, M.G. Pedersen and K.G. Symms: Hydroxylation of 3,4-benzpyrene in human fetal tissue homogenates. *Biochem. Pharmacol.* 21: 2269, 1972.
2. K.G. Symms and M.R. Juchau: Mechanism of aromatic nitro group reduction in the soluble fraction of human placenta. *Biochem. Pharmacol.* 21: 2519, 1972.
3. M.R. Juchau, Q.H. Lee and P.M. Blake: Inverse correlation between aryl hydrocarbon hydroxylase activity and conversion of cholesterol to pregnenolone in human placentas at term. *Life Sci.* 11: 949, 1972.
4. M.R. Juchau and M.G. Pedersen: Drug biotransformation reactions in the human fetal adrenal gland. *Life Sci.* 12: 193, 1973.
5. M.R. Juchau and E.A. Smuckler: Subcellular localization of human placental aryl hydrocarbon hydroxylase. *Toxicol. Appl. Pharmacol.* (In press, 1973).

1003542252

Abstracts (Cont'd):

12. Tabei, T., and Heinrichs, W.L.: Puberty and hepatic 16-oxygenation of 3β -hydroxyandrost-5-en-17-one (DHA). The 53rd Annual Meeting of The Endocrine Society, San Francisco, 1971.
13. Heinrichs, W.L., Haga, H., and Tabei, T.: Progesterone 5 α -reductase activity in cell-free homogenates of rat brain and other tissues. The 19th Annual Meeting of The Society for Gynecologic Investigation, San Francisco, 1972, No.75.
14. Omenn, G.S., Figley, M.M., and Heinrichs, W.L.: Radiographic intrauterine diagnosis for the TAR syndrome (thrombocytopenia with absent radii). Presented at the Annual Meeting, American Society of Human Genetics, Philadelphia, 1972. Am. J. Hum. Genet. 24: 31a, 1972.
15. Tabei, T., Haga, H., Heinrichs, W.L., and Herrmann, W.L.: Metabolism of progesterone by the brain and the pituitary gland. Clin. Res. 21: 206, 1973.

1003542238

16

CURRICULUM VITAE

Toru Tabei, M.D., Ph.D.

*Council for Tob.
Research*Personal Data:

Birth Date
Birth Place
Citizenship
Marital Status
Children

REDACTED

REDACTED

Education:

High School	Kumagaya High School, Japan	1951-54
B.S.	Chiba Governmental College Chiba, Japan	1954-57
M.D.	University of Chiba School of Medicine Chiba, Japan	
Rotating Intern	Toranomon Hospital Tokyo, Japan	1961-62
Resident; and Post-residency training program (Ph.D.)	Department of Obstetrics & Gynecology University of Tokyo Tokyo, Japan	1962-66
Senior Fellow	Department of Obstetrics & Gynecology University of Washington School of Medicine Seattle, Washington	1970-71

Academic Appointments:

Research Associate	Montefiore Hospital Department of Medicine University of Pittsburgh Pittsburgh, Pennsylvania	1966-68
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1003542240

BIBLIOGRAPHY

Toru Tabei, M.D., Ph.D.

1. Nakayama, T., Arai, K., Satoh, K., Nagatomi, K., Tabei, T., and Yanaihara, T.: The formation of estriol from estradiol-17 β by the human fetal adrenal tissue. *Endocrinologia Japonica* 13: 153, 1966.
2. Nakayama, T., Arai, K., Yanaihara, T., Tabei, T., Satoh, K., and Nagatomi, K.: Oestrogen metabolism in anencephalus. *Acta Endocrinologica* 55: 369, 1967.
3. Nakayama, T., Arai, K., Tabei, T., Yanaihara, T., Satoh, K., and Nagatomi, K.: Biosynthesis of estrogens in *in vitro* perfusion of the human placenta. *Endocrinologia Japonica* 14: 251, 1967.
4. Nakayama, T., Arai, K., Nagatomi, K., Satoh, K., Tabei, T., Yanaihara, T., and Fujita, Y.: Biosynthesis of estrogens by the perfused human ovary. I. Conversion of progesterone-14C and androst-4-ene-3, 17-dione-14C to estradiol-17 β . *Endocrinologia Japonica* 14: 259, 1967.
5. Nakayama, T., Arai, K., Yanaihara, T., Satoh, K., Nagatomi, K., Tabei, T., and Fujita, Y.: Formation of estrogens in the feto-placental compartments. *Endocrinologia Japonica* 15: 135, 1968.
6. Tabei, T.: Biosynthesis of estrogens in the human placenta. (Thesis presented to the Department of Obstetrics and Gynecology, University of Tokyo). *Acta Obstetrica et Gynaecologica Japonica* 17: 1, 1970.
7. Tabei, T., and Heinrichs, W.L.: Enzymatic oxidation and reduction of C₁₉- Δ^5 -3 β -hydroxysteroids by hepatic microsomes. I.. Biosynthesis of 3 β , 17 β -dihydroxyandrost-5-16-one and sex differences in adult rats. *Endocrinology* 91: 969, 1972.
8. Tabei, T., and Heinrichs, W.L.: Enzymatic oxidation and reduction of C₁₉- Δ^5 -3 β -hydroxysteroids by hepatic microsomes. II. Effect of age in rats on 16, 17-oxido-reduction of 3 β -hydroxyandrost-5-en-17-one (DHA). *Endocrinology* 92: 1161, 1973.

1003542242

Curriculum Vitae - Felix G. Freund, M.D.

Page 2

Board Certification:

1962 . Diplomate, American Board of Anesthesiology

Licensure to Practice:

1958	Iowa
1962	Missouri
1963	Washington

Organizations:

REDACTED

REDACTED

1003542266

3.

13. Budget for the coming year:

A. Salaries (give names or state "to be recruited"):

Professional (give % time of investigator(s)
even if no salary requested)

% time

Amount

Edward R. Bowman, Ph.D. Research associate 100
Faye J. Bowman, Ph.D. Research associate 10022,846
15,952

Technical

Kenneth Wilson, M.S.

90

8,527

4.46000
5000
3100

Sub-Total for A 47,325

B. Consumable supplies (by major categories)

Chemicals and reagents
Laboratory glassware
Animals, animal care and feed
Isotopes3,500
1,500
3,800
1,600

Sub-Total for B 10,400

C. Other expenses (itemize)

Reprint and page charges
Clerical (including typing)
Travel (papers at meetings, conferences, etc.)400
800
800

Sub-Total for C 2,000

Running Total of A + B + C 59,725

D. Permanent equipment (itemize)

Updating (modernization) of mass spectrograph

5,500

Sub-Total for D 5,500

E 8,959

E. Indirect costs (15% of A+B+C)

Total request \$74,184

1003542100

THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

August 1, 1973

Grant Application No. 932
MISCELLANEOUS

To: The committee comprising Drs. Bing, Loosli, and Sommers

Subject: W. LeRoy Heinrichs, M.D., Ph.D., University of Washington
New application No. 932
"The Effect of Smoking on Adaptive Changes of Previabale Human Pregnancies"

History

This proposal originally reached us as a "Case". A delay ensued because of discussions about indirect cost rates. Since our July 31 closing date was then imminent, and as the program relevance for CTR seemed apparent, we gave Dr. Heinrichs the option to apply formally, without commitment, of course.

The request is for \$73,108 plus two additional years.

Documents Submitted

Attached is application dated 7-20-73 with C.V.'s of Drs. Heinrichs, Tabei, Conrad, Juchau, Shepard, Schiller, Freund, and Vontver.

Reprints of the recent papers listed are here, and will be forwarded if you request.

Comment

This investigation involves the controversial question of use of aborted fetuses in research. Dr. Heinrichs writes that approval by his local committee is not available yet, but that he has every reason to believe approval will be forthcoming.

F.W.N.
F.W.N.

FWN:wg
Encl.

1003542218

CURRICULUM VITAE

Harvey S. Schiller, M.D.

Personal Data:

Birth Date:
Birth Place:
Citizenship:
Marital Status:
Children:

REDACTED

Education:

B.S.	University of Wisconsin Madison, Wisconsin	1959-62
M.D.	Washington University St. Louis, Missouri	1962-66
Intern	Department of Pathology Yale University School of Medicine New Haven, Connecticut	1966-67
Resident; and Postdoctoral Fellow	Department of Pathology Yale University School of Medicine New Haven, Connecticut	1967-68
Postdoctoral Fellow	Department of Laboratory Medicine Yale University School of Medicine New Haven, Connecticut	1970-71

Faculty Appointments:

Chief Resident; and Instructor	Department of Laboratory Medicine Yale University School of Medicine New Haven, Connecticut	1971-72
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1003542261

BIBLIOGRAPHY

William LeRoy Heinrichs, M.D., Ph.D.

1. Heinrichs, W.L.: Studies of serum proteins and glycoproteins in monozygotic (MZ) and dizygotic (DZ) twins. Thesis submitted to the Student Research Achievement Committee, University of Oklahoma School of Medicine, 1958.
2. Heinrichs, W.L. and M.R. Shetlar: Serum glycoproteins in monozygotic and dizygotic twins. *Proc. Soc. Exp. Biol. & Med.* 99: 132, 1958.
3. Heinrichs, W.L.: Soft tissue dystocia. *Harper Hosp. Bull.* 19: 80, 1961.
4. Heinrichs, W.L., Climie, A.R.W. and Cook, J.C.: Cure of primary mesodermal mixed tumor by radiotherapy. *Obstet. & Gynec.* 19: 537, 1962.
5. Heinrichs, W.L., Kommesser, J.G. and Tullock, J.A.: Continuous lumbar epidural anesthesia in obstetrics. *Harper Hosp. Bull.* 20: 6, 1962.
6. Heinrichs, W.L.: The neutrophile alkaline phosphatase test in pregnancy. *Harper Hosp. Bull.* 20: 107, 1962.
7. Heinrichs, W.L.: Pelvic hematomas following delivery. *Harper Hosp. Bull.* 21: 56, 1963.
8. Climie, A.R.W., Heinrichs, W.L., and I.J. Foster: Neutrophilic alkaline phosphatase test: A review with emphasis on findings in pregnancy. *Tech. Bull. Regis. Med. Tech.* 32: 95, 1962.
9. Colás, A., Heinrichs, W.L., and Tatum, H.J.: Pettenkofer chromogens in the maternal and fetal circulations: detections of 3β , 16α -dihydroxyandrost-5-en-17-one in umbilical cord blood. *Steroids* 3: 417, 1964.
10. Colás, A., and Heinrichs, W.L.: Pettenkofer chromogens in maternal and fetal circulations: anencephalic pregnancies, cesarean sections and tentative identification of 3β , 17β -dihydroxyandrost-5-en-16-one in umbilical cord blood. *Steroids* 5: 753, 1965.
11. Heinrichs, W.L.: A method for the analysis of specific urinary 3β -hydroxy- Δ^5 -steroids and some applications in clinical medicine. Thesis presented for the Master of Science Degree in Biochemistry, University of Oregon Medical School, 1965.
12. Heinrichs, W.L., Feder, H.H., and Colás, A.: The steroid 16α -hydroxylase system in mammalian liver. *Steroids* 7: 91, 1966.
13. Heinrichs, W.L., Mushen, R.L., and Colás, A.: The 7β -hydroxylation of 3β -hydroxyandrost-5-en-17-one by hepatic microsomes. *Steroids* 9: 23, 1967.

1003542234

Curriculum Vitae

THOMAS HILL SHEPARD

May 1973

Place and Date of Birth:Home Address:

REDACTED

Marital Status:Education:

1945	A.B. Amherst College
1948	M.D. University of Rochester
1946-47	Fellowship in Bacteriology and Pathology, University of Rochester
1948-51	Clinical House Staff Training in Pediatrics
1951-52	Chief Resident, Pediatrics, and Instructor in Pediatrics, University of Rochester
1953	American Board of Pediatrics
1954-55	Fellow in Endocrinology, Johns Hopkins Medical School (under Dr. Lawson Wilkins)

Military Service:

1952-54	Captain, M.C., Air Force
---------	--------------------------

Positions Held:

1955-56	Instructor in Pediatrics, University of Washington, School of Medicine
1956-61	Director of Endocrinology, Children's Orthopedic Hospital, Seattle, Washington
1956-61	Assistant Professor of Pediatrics, University of Washington, School of Medicine
1961-62	Research Associate in Embryology, Department of Anatomy and Visiting Assistant Professor of Pediatrics, College of Medicine, University of Florida
1962-68	Associate Professor of Pediatrics, University of Washington, School of Medicine
1962	Visiting Investigator, Department of Embryology, (6 mo.) Carnegie Institution of Washington, Baltimore, Maryland
1963	Visiting Investigator, Fetal Laboratory, Department of Pediatrics (6 mo.), University of Copenhagen, Denmark
1964- Present	Head, Central Laboratory for Human Embryology

1003542253

Wattenberg, et al. (1962) as described by Juchau (1971). Cholesterol sidechain-cleavage will be determined by a modification of the methods of Morrison, et al. (1965) as described by Juchau, et al. (1972). The assay method for androgen aromatization utilizes liquid scintillation spectrometry, and will be performed by modifications (Juchau, et al. 1972) of the methods described by Shaw and Dalziel (1969). Azo dye N-demethylase activities will be studied utilizing the method described by Welch, et al. (1968). The ω -oxidation of laurate will be assayed according to the method of Kusunose, et al. (1964). Glucuronyl transferase will be assayed according to methods described by Dutton (1963). Naphthylamine N-hydroxylase activities will be determined according to the methods described by Ziegler, et al. (1973) and procaine esterase will be assayed by the method of Kalow (1952).

Fetoplacental Enzymology (Obstetrics and Gynecology)

The dehydroepiandrosterone oxido-reductase activities of liver will be carried out according to procedures described by Tabei and Heinrichs (Endocrinol., Vol. 91, p. 967, 1972; Vol. 92, p. 1161, 1973; and Endocrinol., in press.). These procedures for quantitation of progesterone 5 α reductase and 20 α hydroxysteroid dehydrogenase activity in fetal brain utilize ^{14}C -4-progesterone and quantitation with liquid scintillation spectrometry. Data will be expressed as specific activity of enzymes, picamoles hour $^{-1}$ milligram $^{-1}$. Protein will be determined by the Lowry procedure (Tabei T., Haga H., Heinrichs W.L. and Herrmann W.L. Submitted to Steroids, 1973).

1003542224

Abstracts:

1. Freund, F.G., and de Jong, R.H.: Earliest evidence of phase II myoneural block. *Anesthesiology* 28:250, 1967.
2. Freund, F.G., Hornbein, T.F., Martin, W.E., and Parmentier, P.: Effect of halothane and halothane-nitrous oxide on the H-reflex in man. *Anesthesiology* 29:191, 1968.

1003542271

10. Space and facilities available (when elsewhere than item 2 indicates, state location):

The Pregnancy Termination Center of the Department of Obstetrics and Gynecology is directed by Dr. Louis Vontver at Harborview Medical Center, Seattle. It is staffed with obstetricians, anesthesiologists, nurses, and social workers active in terminating approximately 250 pregnancies annually. Physician staffing is contributed by clinical faculty working under the supervision of Dr. Vontver, who will coordinate the clinical management.

Cardiopulmonary studies will be performed in the Clinical Research Center of Harborview Medical Center under the direction of Dr. Felix Freund, member of the Anesthesia Research Center, University of Washington School of Medicine. Equipment described in the appended article by Wong, et al., Anesthesiology, Vol. 38, p. 542 (reprints - Dr. Felix Freund) will be utilized.

The Central Laboratory for Human Embryology is a completely equipped and fully staffed research laboratory of approximately 2,400 square feet in several rooms in the Child Development and Mental Retardation Center adjacent to the University Hospital. A portion of the time for professional staff for examination of the material is requested for this laboratory. Dissecting microscopes, instruments and several types of balances are available for this work. The laboratory is fully equipped for performing the histologic studies.

The Physiological Studies of Umbilical Cords will be carried out in the laboratories of Dr. John Conrad, Department of Obstetrics and Gynecology, University Hospital in facilities (see attached)

11. Additional facilities required:

none

12. Biographical sketches of investigator(s) and other professional personnel (append):

13. Publications: (five most recent and pertinent of investigator(s); append list, and provide reprints if available).

1003542225

Page 3a.

Item 10, continued

The Lipid and Hormone Assays will be carried out in the Cost Center of the Department of Laboratory Medicine, and in laboratories of the Department of Obstetrics and Gynecology by Dr. Louis Vaniver at Harborview Medical Center, Seattle. It is situated with facilities, including 625 square feet of working space, and containing two eight-channel recorders (one model HP 7700 and one Offner, type R) and one two-channel BLH recorder; five Sensitive Force Endevco 8107.2 transducers for small muscle studies; five Medium Force transducers; thermostatically regulated baths for up to eight test chambers; and one test station for sucrose gap potential measurement. The smaller microelectrode laboratory has 300 square feet for working space and contains two two-channel oscilloscopes (Tectronix 502); three negative capacitance preamplifiers; one four-channel paper recorder (Sanborn 150); one analog computer; one electronic stimulator (AEL 104A); time and signal generators; one oscilloscope camera (35 mm.); Leitz micromanipulators; Prior micromanipulators; Kopf micro-electrode puller and dissection microscopes.

The Lipid and Hormone Assays will be carried out in the Cost Center of the Department of Laboratory Medicine, and in laboratories of the Department of Obstetrics and Gynecology by Dr. Harvey Schiller. A 600 square foot Endocrinology Laboratory, providing hormone assays for the University Hospital is located within the Department of Obstetrics and Gynecology and will be the site of these quantitative estimates. This laboratory and an adjacent instrument room are equipped with spectrophotometers, Barbara Coleman model 10 gas liquid chromatographs, Packard model 6240 Liquid Scintillation Spectrometers and strip scanners, water baths, evaporative extractors, ventilated hoods, etc.

Fetoplacental Enzymology (Pharmacology) will be completed in facilities comprising one biochemical pharmacology laboratory of approximately 600 square feet, with ready access to two other well equipped biochemical laboratories in the immediate vicinity. These include approximately 1,700 square feet; 330 square feet of additional laboratory space also has been acquired recently. A cold room with facilities for enzyme isolation and tissue preparation (immediately adjacent) will also be available.

The major pertinent items of equipment available for the proposed research project include:

1. Spinco Model L ultracentrifuge
2. Beckman DU spectrophotometer
3. Gilford Model 2000 recording spectrophotometer with constant temperature and automatic sample changer attachments and recorder
4. Beckman Expandomatic pH meter
5. Beckman DB spectrophotometer
6. Liquid scintillation counter - Nuclear Chicago, Mark I
7. Buchler Flash evaporator
8. Three Dubnoff shaking incubators; one Burrel shaking machine; assorted water baths and centrifuges
9. Constant temperature, explosion-proof thin layer chromatography oven
10. Mettler Precision balance; Mettler top-loader balance
11. Automatic glassware washer

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In a portion of the experiments, rats will be pretreated with reserpine before sacrifice. After the rest period, vas deferens will be exposed to guanethidine and indirectly acting sympathomimetic agents, as described above, before they are assayed for guanethidine.

(3) ^{14}C -norepinephrine experiments: Interaction of indirectly acting sympathomimetic agents with ^{14}C -norepinephrine in isolated smooth muscle preparations will be carried out with rat vas deferens and rabbit aortic strips.

Aortic segments will be obtained from rabbits sacrificed by air embolism. The thoracic aorta will be cut into helical strips according to the method of Furchgott (Methods Med. Res. 8:117,1960). Each helical strip is then cut into four segments and tissues mounted on stainless steel rods under tension and allowed to equilibrate for one hour in Tyrode solution before they are transferred to a reservoir containing Tyrode solution and ^{14}C -norepinephrine ($5.2 \times 10^{-7}\text{M}$, $0.1 \mu\text{Ci/ml}$) for an additional 20-30 minute period. This time period has been selected on the basis of previous experiments conducted in this laboratory which revealed that within 20 minutes the amount of ^{14}C -norepinephrine in the tissue is increasing and the amount of radioactivity in the form of ^{14}C -norepinephrine metabolite remains constant.

The effects of sympathomimetics on the accumulation of ^{14}C -norepinephrine will be assessed by adding the agent under study for the last 30 minutes of the rest period and before the addition of isotope. Accumulation of radioactivity by aortic strips must be assessed in reserpine pretreated and control preparations in order to eliminate alterations in turnover rate of transmitter stores in sympathetic nerve endings from obscuring the results.

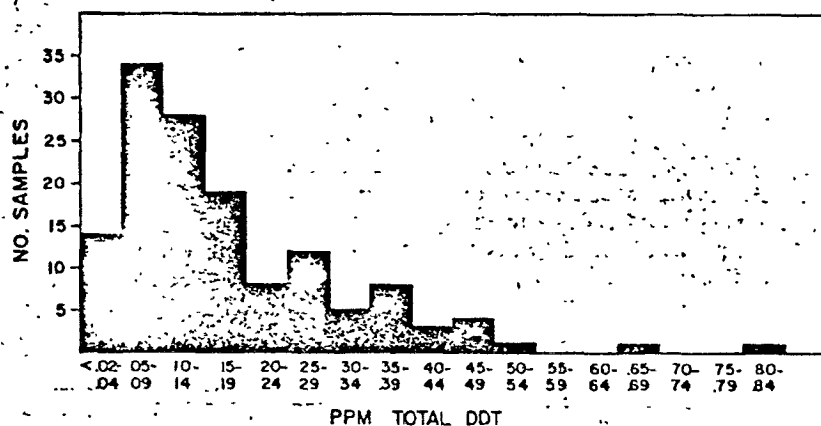
After the incubation of tissues in radioactive norepinephrine they are washed, blotted and weighed. Tissues are placed in test tubes containing 1 N NaOH for 16 hours with shaking. This solution dissolves the tissue. Alkalinity is neutralized by the addition of an equal volume of 1 N HCl and aliquots evaporated in aluminum planchets for counting of radioactivity. Salt quenching is minimized by adding an identical volume of 1 N NaCl to all planchets containing aliquots of the reservoir. ^{14}C -norepinephrine content of tissues is expressed as a ratio of cpm/g tissue to cpm/ml reservoir (tissue to medium ratio).

^{14}C -norepinephrine accumulation by rat vas deferens will be assayed in experiments similar to those described above. After a 15-20 minute exposure to radioactive norepinephrine, tissues will be removed, washed, blotted and weighed. Barnette et al (Brit. J. Pharmacol. 34:484,1968) has shown that at this time period most of the radioactivity in the vas deferens is in the form of unchanged norepinephrine. Tissue accumulation of isotope will be expressed as a tissue to medium ratio.

The action of indirectly acting sympathomimetic agents on ^{14}C -norepinephrine taken up by tissues can be assessed by transferring tissues from the reservoir into nonradioactive Tyrode solution at 2 minute intervals for one hour. Preliminary experiments have demonstrated that the half time for desaturation of radioactivity is approximately 35 minutes. This experimental design can be used to compare equipotent concentrations of indirectly acting sympathomimetic agents on release of recently captured ^{14}C -norepinephrine. The drug under study can be added to the washout medium and the slope of desaturation curves compared to reveal differences in potency. Combinations of sympathomimetic agents can be compared with individual agents by this method.

Data will be expressed as percent of radioactivity remaining in the tissue at each time period and comparisons between drug treatments will be made by linear regression analysis. All statistical analyses will be conducted according to the methods described in Steel and Torrie (1960).

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Frequency distribution of human milk samples by concentration of total DDT.

Table 1.—Total DDT Concentrations in Human Milk by City of Origin

City	No. Samples	Mean, ppm	Standard Deviation	P*
Long Island	14	0.100	0.10	.01
Rochester	20	0.17	0.13	NS†
Chicago	19	0.18	0.10	NS
Lexington	27	0.22	0.17	NS
Nashville	34	0.17	0.15	NS
Memphis	6	0.15	0.08	NS
Los Angeles	18	0.18	0.12	NS

* Probabilities of the difference between the corresponding mean and the mean of the total population being due to chance.

† Not significant.

Table 2.—Home Pesticide Use, Exterminator Use, and DDT in Human Milk

	No. Samples	Mean, ppm	Standard Deviation
Home Use			
Does not use pesticides	81	0.17	0.14
Uses pesticides	52	0.18	0.14
Exterminator Use			
No exterminators	107	0.18	0.15
Uses exterminators	30	0.14	0.10

Results

A total of 138 samples of human milk from 101 donors was analyzed. The mean total DDT concentration was 0.17 ppm with a standard deviation of 0.14 ppm. The range of concentrations was from less than 0.02 ppm to 0.83 ppm (Figure). Only four specimens had undetectable concentrations of total DDT (< 0.02 ppm) and three had concentrations in excess of 0.50 ppm.

The results are presented according

to geographic area in Table 1. The mean concentration of the samples from the Long Island communities (0.10 ppm) is significantly lower than those from the other cities ($P = .01$). The mean of the Lexington samples exceeded that from the other areas, but this difference did not achieve statistical significance.

Of particular interest was the relationship of total DDT concentrations to prior reported exposure to pesticides (Table 2). Of the women, 39% had used pesticides in their homes or

Table 3.—DDT in Human Milk and Use of Butter or Margarine

	No. Samples	Mean, ppm	Standard Deviation
Butter	31	0.14	0.10
Margarine	40	0.20	0.16

Table 4.—Total DDT Concentrations (ppm) in Matched Fore-Milk and Hind-Milk Samples

Fore-Milk	Hind-Milk	Difference
0.09	0.13	+0.04
0.04	0.12	+0.08
0.06	0.16	+0.10
0.13	0.26	+0.13
0.03	0.06	+0.03
0.04	0.08	+0.04
0.23	0.35	+0.12
0.10	0.21	+0.11
0.05	0.17	+0.12
0.29	0.37	+0.08
0.11	0.12	+0.01
0.14	0.16	+0.02
0.68	0.45	-0.23
0.29	0.52	+0.23
0.11	0.35	+0.24
0.06	0.09	+0.03
0.06	0.46	+0.40
0.16	0.11	-0.05
0.13	0.15	+0.02
0.07	0.09	+0.02
0.16	0.04	-0.12
0.06	0.18	+0.12
0.37	0.48	+0.11
0.03	0.24	+0.21

gardens, but there was not a statistically significant difference in the total DDT concentrations in the milk from the two groups. However, when those women employing exterminators were compared with those who used pesticides on their own, it was found that exterminator use was associated with lower concentrations of total DDT in the milk ($P = .05$). Frequent pesticide exposures at some time in the past, usually associated with agricultural activities, were reported by 26 women. The concentrations of total DDT in their milk did not differ significantly from those who were not so exposed.

Regarding diet, no significant correlations could be found between total DDT concentrations in milk and

4. Friedman, G.D., Siegelau, A.B., and Seltzer, C.C.: Cigarette smoking and exposure to occupational hazards. To be published in the American Journal of Epidemiology.
5. Oakes, T.W., Friedman, G.D., and Seltzer, C.C.: Mail survey response by health status of smokers, non-smokers, and ex-smokers. To be published in the American Journal of Epidemiology.
6. Oakes, T.W., Friedman, G.D., Seltzer, C.C., Siegelau, A.B., and Collen, M.F.: Health services utilization by smokers and non-smokers. To be published in Medical Care.

Papers Submitted for Publication

7. Dales, L.G., Friedman, G.D., Siegelau, A.B., and Seltzer, C.C.: Cigarette smoking and serum chemistry tests.
(some of the findings in this paper were presented at the American Heart Association's Conference on Cardiovascular Epidemiology, New Orleans, March 13, 1973).
8. Siegelau, M.S., Friedman, G.D., Adour, K., and Seltzer, C.C.: Hearing loss in adults: Relation to age, sex, exposure to loud noise and cigarette smoking.

Reprints or drafts of each of the above have been submitted to Dr. Hockett, mostly accompanying a letter of February 28, 1973.

Papers Nearly Completed (approximate titles)

9. Seltzer, C.C., et al: Smoking habits and pain tolerance.

Currently in draft form, this paper shows that among white men and women, and black women, smokers show a lower pain tolerance than non-smokers.

10. Dales, L.G., et al: Racial differences in serum and urine glucose after glucose challenge.

This paper is a report of striking racial differences in serum glucose. While not primarily a comparison of smokers and non-smokers, these findings resulted from our basic smoker-nonsmoker analyses and were deemed a sufficiently important contribution to be written up and published. This should be ready shortly for submission for publication.

11. Friedman, et al: Cigarettes, alcohol, coffee and peptic ulcer.

While cigarette smoking has been associated with peptic ulcer in a number of studies and in our data as well, no one to our knowledge has tried to determine whether alcohol and coffee -- which increase gastric acid secretion and which are associated with cigarette smoking -- could account for the smoking-ulcer relationship. This analysis and report are nearly completed.

ABSTRACTS

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2. Heinrichs, W.L., and Colas, A.: Quantitation of urinary dehydroepiandrosterone 16α -hydroxydehydroepiandrosterone and other 3β -hydroxy- Δ^5 -steroids in pregnancy and hirsutism. Androgens in Normal and Pathological Conditions. Proc. 2nd Symp. on Steroid Hormones. June, 1965, Ghent, Belgium.; p. 62, Excerpta Medica Foundation, New York, 1966.
3. Heinrichs, W.L., and Colas, A.: Hepatic microsomal hydroxylations of dehydroepiandrosterone (DHA). 49th Meeting of The Endocrine Society, 211: 134, 1967.
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5. Heinrichs, W.L., Kahwanago, I., and Herrmann, W.L.: Binding of ^3H -estradiol by 'receptors' in bovine hypothalamus and pituitary gland, and inhibition by clomiphene citrate. 16th Annual Meeting of the Society for Gynecologic Investigation, Denver, Colo., 1969.
6. Farber, M., Conrad, S., Heinrichs, W.L., and Herrmann, W.L.: Binding of estradiol by normal and neoplastic human myometrium. The 51st Annual Meeting of The Endocrine Society, New York, 164: 112, 1969.
7. Depp, R., Pion, R.J., and Heinrichs, W.L.: Inhibition of pregnenolone 3β -hydroxy-steroid dehydrogenase system in human placenta and corpus luteum of pregnancy. The 51st Annual Meeting of The Endocrine Society, New York, 211: 136, 1969.
8. Kahwanago, I., Heinrichs, W.L., and Herrmann, W.L.: Species and age differences in estradiol 'receptors'. The District VIII Annual Meeting of the American College of Obstetricians and Gynecologists, Albuquerque, New Mexico, 1969.
9. Youatt, G., Wyss, H.I., Heinrichs, W.L., and Herrmann, W.L.: Binding of pregnenolone in cytoplasm from rat and dog prostate. The 52nd Annual Meeting of The Endocrine Society, St. Louis, 1970.
10. Wyss, H.I., Youatt, G., Heinrichs, W.L., and Herrmann, W.L.: Influence of magnesium on the stability of the estradiol 'receptor' in uterine cytoplasm. The 52nd Annual Meeting of The Endocrine Society, St. Louis, 1970.
11. Heinrichs, W.L.: Oxidative metabolism of steroids and drugs by hepatic microsomes from pregnant rats. Presented at the 17th Annual Meeting of the Society for Gynecologic Investigation, New Orleans, 1970.

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26. "Exposure of Susceptible Teachers to Rubella Vaccinees," W. F. Fleet, W. Schaffner, L. B. Lefkowitz, G. D. Murphy, and D. T. Karzon, A. J. Dis. Child., 123, 28 (1972).
27. "Botulism," Chapter in The Science and Practice of Clinical Medicine, edited by J. Dietschy, et al, Grune and Stratton, Inc., New York (in press) (W. Schaffner).
28. "The Postoperative Detection of Salmonella typhi: An Unexpected Hospital Infection Hazard," G. Reisig and W. Schaffner, Arch. Surg., 104, 349 (1972).
29. "Microbiological Safety of Solutions and Delivery to the Patient: Problems in Preparation and Handling," W. Schaffner, In Proceedings of the Symposium on Total Parenteral Nutrition, Nashville, Tenn., Jan. 17-19, 1972. Food Science Committee, Council on Foods and Nutrition of the American Medical Association, pp 126-131.
30. "Topics in Infectious Diseases: Current Antibiotic Sensitivities of Gram-negative Bacteria," W. Schaffner, H. B. Ratner, and M. G. Koenig, J. Tenn. Med. Association, 65, 615 (1972).
31. "Rocky Mountain Spotted Fever. In Current Therapy - 1973," W. Schaffner, edited by H. F. Conn, W. B. Saunders Co., Philadelphia, Pa., (in press).
32. "Topics in Infectious Diseases: Increasing Resistance of Shigellae to Antibiotics," R. L. Harbin, H. B. Ratner, and W. Schaffner, J. Tenn. Med. Association, 65, 999 (1972).
33. "Sepsis Due to Contaminated Intravenous Fluids: Epidemiologic, Clinical, and Laboratory Observations in One Hospital," S. K. Felts, W. Schaffner, M. A. Melly, and M. G. Koenig, Ann. Int. Med., 77, 881 (1972).
34. "Septicemia and Total Parenteral Nutrition: Distinguishing Catheter-related from Other Septic Episodes," J. D. Dillon, W. Schaffner, C. W. VanWay, and H. C. Meng, J. A. M. A., (in press).
35. "DDT Levels in Human Milk," D. J. Wilson, D. J. Locker, C. A. Ritzen, J. T. Watson, and W. Schaffner, Am. J. Dis. Child., 125, 814 (1973).
36. "Infection of an Avulsed Papillary Muscle Tip Simulating Bacterial Endocarditis," T. K. Satterwhite, Z. A. McGee, W. Schaffner, G. C. Friesinger, M. Mishu, and R. D. Collins, Am. Heart J. (in press).
37. "Septicemia Associated with Scalp-vein Needles," R. L. Harbin and W. Schaffner, South. Med. J. (in press).
38. "Hospital-acquired Infections," Chapter in Infectious Diseases in Obstetrics and Gynecology, edited by G. R. G. Monif, Hoeber Medical Division, Harper and Row Publishers, Inc., Hagerstown, Md., (in press) (W. Schaffner).

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PUBLICATIONS OF WILLIAM SCHAFFNER, M.D.

1. "The Diarrhea of Travelers. V. Prophylaxis with Phthalysulfathiazole and Neomycin Sulfate," B. H. Kean, W. Schaffner, R. W. Brennan, and S. R. Waters, J. A. M. A., 180, 367 (1962).
2. "The Diarrhea of Travelers. IV. Viral Studies of Visiting Students in Mexico with Further Bacteriologic and Parasitologic Observations," M. A. Rosenbluth, W. Schaffner, and B. H. Kean, Am. J. Trop. Med., 12, 239 (1963).
3. "Thrombocytopenic Rocky Mounty Spotted Fever: Case Study of a Husband and Wife," W. Schaffner, A. C. McLeod, and M. G. Koenig, Arch. Int. Med., 116, 857 (1965).
4. "Fatal Pneumonia Due to a Tetracycline-resistant Pneumococcus," W. Schaffner, W. M. Schreiber, and M. G. Koenig, New Eng. J. Med., 274, 451 (1966).
5. "Bacterial Interference in the Therapy of Recurrent Staphylococcal Infections: Multiple Abscesses due to the Implanation of the 502A Strain of Staphylococcus," D. J. Drutz, M. H. VanWay, W. Schaffner, and M. G. Koenig, New Eng. J. Med., 275, 1161 (1965).
6. "Type B Botulism in Man," M. G. Koenig, D. J. Drutz, A. I. Mushlin, W. Schaffner, and D. E. Rogers, Amer. J. Med., 42, 208 (1967).
7. "Lysostaphin: An Enzymatic Approach to Staphylococcal Disease. I. In Vitro Studies," W. Schaffner, M. A. Melly, J. H. Hash, and M. G. Koenig, Yale J. Biol. Med., 39, 215 (1967).
8. "Lysostaphin: An Enzymatic Approach to Staphylococcal Disease. II. In Vivo Studies," W. Schaffner, M. A. Melly, and M. G. Koenig, Yale J. Biol. Med., 39, 230 (1967).
9. "The Clinical Spectrum of Endemic Psittacosis," W. Schaffner, D. J. Drutz, G. W. Duncan, and M. G. Koenig, Arch. Intern. Med., 119, 433 (1967).
10. "The Penetration of Penicillin and Other Antimicrobials into Joint Fluid. Three Case Reports with a Reappraisal of the Literature," D. J. Drutz, W. Schaffner, W. J. Hillman, and M. G. Koenig, J. Bone Joint Surg., 49, 1415 (1967).
11. "Infection Following Cardiovascular Surgery: Clinical Study Including Examination of Antimicrobial Prophylaxis," J. S. Goodman, W. Schaffner, H. A. Collins, E. J. Battersby, and M. G. Koenig, New Eng. J. Med., 278, 117 (1968).
12. "The Rickettsioses," Chapter 25 in Dermatology in General Medicine, edited by T. B. Fitzpatrick, et al, New York, McGraw-Hill Book Co., 1971, pp 1845-1853.
(D. E. Rogers and W. Schaffner).

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Page 2

Thomas Hill Shepard

<u>Positions Held:</u>	1968-	Professor of Pediatrics, University of Washington, School of Medicine
	Present	
	1967-72	Director of NIH Training Grant in Human Embryology and Teratology
	1968	President - The Teratology Society
	1970	President - Western Society for Pediatric Research
	1971-72	Bureau of Drugs, Food and Drug Administration (Consultant)
	1972-73	National Institute of Child Health and Human Development (Consultant)
	1972-73	Invited-Professor (6 mo.), Department of Pediatrics, University of Geneva
<u>Societies:</u>	Present	
<u>Licensed:</u>	1955	State of Washington

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Thomas Hill Shepard

PUBLICATIONS IN PRESS

1. Bierring F, Shepard TH: Electron microscopic studies on early follicle and colloid formation in the human thyroid. Acta Pathol. Microbiol. Scand.
2. Shepard TH: Embryonic and fetal thyroid development in Endocrine and Metabolic Disorders in Children edited by Vincent Kelley. Paul B. Hoeber, Inc.
3. Shepard TH: Prenatal factors. Ibid.
4. Juchau MR, Pedersen MG, Fantel AG, Shepard TH: Drug metabolism by placenta. Books Clin. Pharmacol. Therap. In press, 1973.
4. Shepard TH: A Catalog of Teratogenic Agents. Johns Hopkins University Press.

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14. First year budget.

A. Salaries (give names or state "to be recruited")
Professional (give % time of investigator(s)
even if no salary requested)

1. David J. Wilson
2. William Schaffner

% time

Amount

20

10

REDACTED

Technical

1. Bruce Ferguson

100

REDACTED

REDACTED

Sub Total for A

B. Consumable supplies (by major categories)

1. Chromatographic supplies and service
2. Chemicals
3. Mass spectrometer analyses
4. Glassware
5. Computer time

350

460

40

200

53

Sub-Total for B

1,103

C. Other expenses (itemize)

None

Sub-Total for C

0

Running Total of A + B + C

REDACTED

D. Permanent equipment (itemize)

None

Sub-Total for D

0

E Indirect costs (15% of A+B+C)

E

1,170

Total request

REDACTED

15 Estimated future requirements

	Salaries	Consumable Suppl	Other Expenses	Permanent Equip	Indirect Costs	Total
Year 2	Not applicable					
Year 3	Not applicable					

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Papers Nearly Completed - continued

12. Seltzer, et al: Responses to psychological questionnaire items by smokers and non-smokers.

The multiphasic health checkup which is the source of our data includes a 155-item psychological questionnaire, derived mostly from the Minnesota Multiphasic Personality Inventory (MMPI). A report of interesting smoker-nonsmoker differences in personality is nearly completed.

13. Seltzer, et al: Differences in pulmonary function related to smoking habits and to race.

Smoker-nonsmoker differences in pulmonary function tests have proven to be less marked in blacks and orientals than in whites. The reasons for these racial differences are being analyzed and a report is nearly complete.

Other Specific Studies currently in progress, with papers likely to result in the near future (approximate titles)

14. Seltzer, et al: Body build in smokers and non-smokers.
15. Dales, et al: Urinalysis results in smokers and non-smokers.
16. Friedman, et al: Smoking and allergy: Analysis of higher prevalence of allergic manifestations in non-smokers than in smokers.

By-Products: Papers Made Possible, at least in part, by C.T.R.-USA Support

Submitted for Publication

17. Friedman, G.D., Siegelaub, A.B., Woodrow, K.M., and Collen, M.F.: Pain Tolerance: Differences between Chinese and Japanese.

This paper is another study which was not directed primarily at smoker-nonsmokers differences but was made possible by the identification of Chinese and Japanese that was carried out for paper No.1 above.

18. Oakes, T.W. and Kodlin, D: On the relation between psychosocial variables and the utilization of medical care.

This paper was made possible by the membership survey partially supported by this grant during the first year.

19. Klatsky, A., Friedman, G.D., and Siegelaub, A.B.: Coffee drinking prior to acute myocardial infarction.

This study was done under an NIH contract, but the paper includes a tabulation of the frequency of coffee drinking in our multiphasic examinee population which had resulted from our CTR-supported data analyses.

14. Heinrichs, W.L.: Hydroxylations of dehydroepiandrosterone by microsomal fractions from mammalian liver. Thesis presented to the Department of Biochemistry and the Graduate Division of the University of Oregon Medical School, in partial fulfillment of the requirements for the degree of Doctor of Philosophy, 1967.
15. Heinrichs, W.L., and Colas, A.: The selective stimulation, inhibition and physico-chemical alteration of the 7- and 16 α -hydroxylases of 3 β -hydroxy-androst-5-en-17-one and drug-metabolizing enzymes. *Biochemistry* 7:2273, 1968.
16. Herrmann, W.L., Spadoni, L.R., and Heinrichs, W.L.: "Androgens and the Gynecologist", chapter in *The Adrenal* (N.P. Christy, ed.) In press.
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22. Heinrichs, W.L., and Colas, A.: Hepatic microsomal 16 α -hydroxylation of 3 β -hydroxyandrost-5-en-17-one (DHA) by fetal, newborn and adult Rhesus monkeys. *Gen. and Comp. Endo.* 14:159, 1970.
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25. Herrmann, W.L., and Heinrichs, W.L. (eds.): "Forum in Obstetrical Practice", *Gynecol. Invest. Suppl. 1*, (S. Karger AG, Basel, Switzerland) 1970.
26. Heinrichs, W.L., Gellert, R.J., Bakke, J.L., and Lawrence, N.: DDT administered to neonatal rats induces persistent estrus syndrome. *Science*, 173: 642, 1971.

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response or the nicotine-response more closely followed the microvascular response of exposure to cigarette smoke.

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Harvey S. Schiller, M.D.

1. Schiller, H.S., and Bensch, K.: De novo fatty acid synthesis and elongation of fatty acids by subcellular fractions of lung. *J. Lipid Research*, 12: 243, 1971.
2. Schiller, H.S., and Donabedian, R.K.: Elongation and esterification of fatty acids in lung by a microsomal fraction. *Am. J. Physiol.*, 224: 1006, 1973.
3. Schiller, H.S., and Donabedian, R.K.: Effect of prostaglandins on fatty acid metabolism in lung. *Biochem. Biophys. Res. Comm.*, 1973. (submitted)

1003542263

LOUISIANA STATE UNIVERSITY MEDICAL CENTER

1100 FLORIDA AVENUE • NEW ORLEANS, LOUISIANA • 70119

DEPARTMENT OF ANATOMY

May 17, 1973

Dr. Robert C. Hockett
The Council for Tobacco Research - U.S.A.
110 East 59th Street
New York, New York 10022

Dear Dr. Hockett:

Please find enclosed a formal application for a research grant entitled, "Microvascular Response of Fetus to Carbon Monoxide or Nicotine." I had submitted a preliminary inquiry last spring (your Case No. 107), but I delayed sending a formal application in order to include some of the results from pilot experiments conducted in my laboratory. Hopefully, these results will help demonstrate the feasibility of the study as well as indicating the type of information that can be derived from the in vivo model I have chosen to use.

I have indicated in the proposal a three year duration for the study; however, I realize that grants are made for one year only and that investigators must re-apply for each subsequent year up to three years.

If any other information is needed by your office or the Scientific Advisory Board, please contact me.

Thank you.

Sincerely,




Sam G. McClugage, Jr., Ph.D.
Assistant Professor

Enclosure

SGM:cfv

APPROVED:



M. D. Woodin, President - LSU System

1003542277

Faculty Appointments (Cont'd):

Assistant Professor	Departments of Laboratory Medicine and Obstetrics and Gynecology (Director, Steroid Section, Chemistry Division, Department of Laboratory Medicine) University of Washington School of Medicine Seattle, Washington	1972 - present
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Military Service:

Captain, U.S. Army	Walter Reed Army Medical Center Armed Forces Institute of Pathology	1968-70
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Specialty Board Certification:

Board Eligible	Anatomic and Clinical Pathology	1972
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COHb. Nicotine has not yet been studied in this regard. In vivo microscopy offers the possibility of measuring vascular dimensions and at the same time observing any changes in the behavior of blood in the microvessels. It is quite conceivable that carbon monoxide and/or nicotine may both alter the dynamic structure and function of the fetal or adult microvascular system which, in turn, will reduce the proper delivery of blood to tissues or organs.

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33. "The Quantum-Dynamics of Anharmonic Oscillators. I. Simple Examples," Elliott B. Alterman, Charlotte M. Tahn, and David J. Wilson, J. Chem. Phys., 44, 451 (1966).
34. "Quantum-Dynamics of Anharmonic Oscillators. II. Systems Having One and Two Degrees of Freedom," Roger C. Baetzold, Charlotte T. Tahn, and David J. Wilson, J. Chem. Phys., 45, 4209 (1966).
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28. "Thermodynamic Functions of More Oscillators," Roger W. Crevelly and David J. Wilson, J. Chem. Phys., 41, 1564 (1964).
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8. Experimental Design and Significance:

A. Experimental Design:

In one experimental group, the mesenteries of fetal and adult pregnant rabbits (New Zealand albino) will be studied. The pregnant rabbits will be anesthetized with Urethane I.V. (ethyl carbamate) or with methoxyflurane using a closed circuit anesthetic machine. To study the fetal circulation, a fetus is exteriorized from the uterus of the mother leaving the placental circulation and fetal membranes intact on various days of gestation between days 25 and 32 and the fetal mesentery is exposed surgically. Homeostasis is maintained by irrigating the field of study with Ringer's solution warmed to the body temperature of the animal by regulating heaters. Gauze sponges covering the fetus and surrounding the mesentery provide insulation for the animal during the experiment. Furthermore, ambient air surrounding the fetus is maintained at bodily temperature (37.5°C) using a Sage air incubator. To study the mesentery of the adult animal, the bowel is displaced after laparotomy and a loop of bowel is exposed. Homeostasis is maintained as in the fetus.

To study the exposed mesentery of the fetus or adult pregnant animal, a beam of monochromatic or white light is brought to the undersurface of the mesentery via a hollow, fused quartz rod; subsequently, the mesentery is transilluminated and examined with a Leitz stereo binocular microscope equipped with 2.5X, 4X, and 10X objectives and 10X and 16X oculars. Measurements of the microvasculature within the mesentery will be performed by a Leitz eyepiece micrometer. Alternatively, the optical images from the microscope will be projected onto the photocathode of a Cohu, RCA, or Fairchild/Dumont vidicon television system and kinerécorded with a Bolex H-16 Rex 5 16mm. motion picture

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6. Brief Description of Objectives or Specific Aims:

There are increasing numbers of reports in the literature which suggest that smoking during pregnancy can cause alterations in the normal development of the fetus in utero. Some of the alterations described are decreased neonatal birth weights, greater incidence of premature delivery, increased incidence of spontaneous abortion, and a higher incidence of stillbirths or neonatal deaths of children born from mothers who smoke. Other authors disagree with the causal relationship between impairment of fetal development and maternal cigarette smoking since they believe that social class, background, and other environmental factors which may affect the mother can have just as profound an effect on the fetus as maternal smoking per se. In the past, many have felt that the nicotine content of cigarette smoke may be the etiologic agent causing alterations in the fetus by possibly crossing the placental barrier. Since nicotine has been demonstrated to have so many pharmacological effects on animals and even man, it was only natural to strongly suspect that it was the harmful agent in cigarette smoke. However, when one examines a list of compounds which have been isolated in the gaseous phase of cigarette smoke, he can readily identify other substances which may too have an effect on a growing fetus in utero. One such compound is carbon monoxide (CO). Cigarette smoke is known to have a relatively high content of CO which in living animals competes with oxygen for binding by hemoglobin. This binding of CO by hemoglobin forms an inactive pigment called carboxyhemoglobin (COHb) which causes a proportional decrease of the oxygen carrying capacity of the blood by shifting the oxyhemoglobin dissociation curve to the left (decreases the unloading tension of oxygen). Since CO is known to cross the placental barrier in various

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animals and man, it may be responsible for the reported effects of maternal smoking on fetal development which heretofore may have been attributed to nicotine. Thus, using an in vivo microscopic method, a study will be conducted in rabbits to observe the responses of the fetal microvascular system after the maternal exposure of carbon monoxide in one group and after the exogenous administration of nicotine to the mother in a second group. The fetal microvascular response in these two experimental groups will then be compared to that of the mother. Exactly how the maternal exposure to cigarette smoke containing the CO and nicotine causes the reported alterations in the fetus has not been adequately described due, in part, to the difficulty in studying in vivo the fetus while maintaining homeostasis. After in vivo observations, tissue samples from the microvessels will be taken in order to prepare them for transmission or scanning electron microscopy. Thus, the in vivo observations can be correlated with tissue sections selected for study by scanning or transmission electron microscopy. This study is designed to specifically examine in vivo the separate effects of nicotine or CO on the fetal microvascular system in an attempt to provide further information on the reported harmful effects of cigarette smoke on the unborn and even adults; thus, the adverse effects of maternal smoking on fetal development and its reported etiologic role in the development of cardiovascular diseases in adults may be better understood.

7. Brief Statement of Working Hypothesis:

Due to previous work done in my laboratory and work done by others, CO may induce alterations in the fetal or adult microvascular system which seriously compromise blood flow to tissues or organs. This reduction of blood flow would seriously reduce the oxygenation of fetal or adult tissues; this, then, would be an additional effect of CO upon the already compromised oxygenation of the blood due to formation of

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Assistant Professor	Department of Physiology and Biophysics University of Washington School of Medicine Seattle, Washington	1962-67
Associate Professor	Departments of Physiology and Biophysics and Obstetrics and Gynecology University of Washington School of Medicine Seattle, Washington	1967-Present

Honors

New York State War Service Scholarship, 1951-55
New York University Scholar, 1961
Visiting Biologist under AIBS/OBE, 1967-71

Labor Foundation Fellow 1968-69

Professional Societies

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Committees

Communications Subcommittee, Medical Illustration and Photography, 1969-70
Perinatal Biology Committee of the Child Development and
Mental Retardation Center, 1969-Present (Chairman, 1973)
Comparative Physiology Training Committee, 1970-Present
Library Advisory Committee, 1972-73
Citizens Advisory Committee for Sunset Community School,
Shoreline School District, 1973
Vestryman and Senior Warden, St. Dunstons Episcopal Church,
Seattle, Washington, 1973

Referee Editor

American Journal of Ob/Gyn
Gynecologic Investigation
Life Sciences
American Journal of Physiology

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THE COUNCIL FOR TOBACCO RESEARCH-U.S.A., INC.

May 30, 1973

Grant Application No. 912

TO: The Committee comprising Drs. Bing, Jacobson and Meier

SUBJECT: Sam G. McClugage, Jr., Ph.D., LSU Medical Center
New Application No. 912
"Microvascular Response of Fetus to Carbon Monoxide or Nicotine"

History

This proposal was case #107, and formal application was encouraged.

Application #912 requests \$38,094. plus two years at lesser amounts

Documents Submitted (attached)

1. Letter dated May 17, 1973 explaining delay in formal application.
2. Application dated May 16, 1973.
3. Reprints:
 - a. "Response of the Fetal Mesenteric . . .", McCuskey, McClugage, Moore and Miller. Proc. Soc. Exptl. Biol. and Med., 132, 636, 1969.
 - b. "In Vivo Microscopic Study of . . .", McClugage and McCuskey. Microvascular Res., 3, 354, 1971.

Comment

If you conclude an outside opinion should be sought, please suggest an appropriate reviewer.

FWN:gh


F.W.N.

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Publications:

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5. McClugage, S. G., and McCuskey, R. S. Microscopic study of the response of the living liver to carbon tetrachloride poisoning. Microvas. Res. 5: 354-360, 1971.
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15. Conrad, J.T. and Kuhn, W.K.: The Active Length-tension Relationship in Human Uterine Muscle. *Am. J. Obstet. & Gynec.* 97:154-160, 1967.
16. Mossman, R. and Conrad, J.T.: The In Vitro Blocking Effect and Oxytocic Effect of the Water-Soluble Estrogens on Pregnant Human, Mouse and Rat Uteri. *Amer. J. Obstet. & Gynec.* 99:539-545, 1967.
17. Tahmoush, Albert, Conrad, J.T., Abrams, R., and Long, E.: The Conduction Velocity of the Action Potential in the In Vitro Rat Uterus as Modified by Estrogens and Pregnancy. (Abstract). *Proceedings of the International Union of Physiological Sciences VII*: 426, 1968.
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19. Dawson, J.E. and Conrad, J.T.: The Effect of Human Chorionic Gonadotropin (HCG) and Luteinizing Hormone (LH) upon the Membrane Potential of Unovulated Frog Oocytes. *The Physiologist* 12:106, 1969.
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21. Conrad, J.T.: The Biophysics of Nidation. The Biology of the Blastocyst. Edited by R.J. Blandau. The University of Chicago Press, 1971.
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23. Dawson, J.E. and J.T. Conrad: "The Effect of Human Chorionic Gonadotropin and Luteinizing Hormone upon the Membrane Potential of Unovulated Frog Oocytes. *Biology of Reproduction* 6:58-66, 1972.

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The proposed program is based on the observation that cigarette smoking during human pregnancies is associated with reduced fetal birthweight at term gestation and produces profound changes in certain oxidative enzymes (aryl hydrocarbon hydroxylase, azo dye N-demethylase and cholesterol sidechain cleavage enzymes) of the placenta. The research involved will examine the hypothesis that the effects of cigarette smoking, and possibly marijuana smoking can produce different effects on these and on several other enzymic activities, and on fetal weight that are already recognizable in previable pregnancies. Any changes in the cardiopulmonary and metabolic-endocrine adjustments of the pregnant female, as they may alter fetal oxygenation via decreasing oxygen saturation or carbon monoxide toxicity, or via nicotine-related decreases in fetal perfusion, may be related to fetal growth and the altered enzyme activities. Those chosen for study are principally mixed function-oxidases utilizing cytochrome P-450 as the terminal oxidase, which requires molecular oxygen for its transformations, and that can be inhibited in vitro by small amounts of carbon monoxide.

9. Details of experimental design and procedures (append extra pages as necessary)

Clinical Subjects

Three groups of women in the first trimester (14-16 weeks) of pregnancy will be solicited for the studies, for which review and approval of the Biomedical Review Committee of the University of Washington will be obtained. Group one, nonsmokers; Group two, women smoking 20-30 cigarettes daily for the duration of the pregnancy; Group three, women regularly smoking marijuana for the duration of the pregnancy. If possible, the cardiovascular studies and blood sampling for metabolic endocrine assessments will be completed on outpatients (Clinical Research Center, approval pending). Abortions will be carried out vaginally after cervical dilatation with laminaria tents, followed by oxytocin infusion and extraction of the fetus, or with the stimulation of uterine contractions by the introduction of an extraovular catheter and infusion of oxytocic substances, followed by expulsion and/or extraction, as clinical management compatible with reasonable fetal tissue integrity necessitates.

Cardiopulmonary Studies

See Wong, et al., Anesthesiology, Vol. 38, p. 542, 1973 (reprints - Dr. Felix Freund) for methods of procedure.

Feto-Placental Morphology

The specimens will be picked up immediately after delivery and after clearance with the pathology department, and brought to the Central Laboratory of Human Embryology in the University Hospital. Fetal blood samples will be obtained as soon as possible. A complete autopsy will be performed, including crown-rump length, weight, crown-head length and the weight of organs. Organs weighed will be the brain, lungs, heart, thymus, thyroid, liver, spleen, adrenal, kidney, placenta and gonad. These measurements will be compared to our own standards (see publication by Tanimura, et al., "Weight Standards for Organs from Early Human Fetuses," 1971). We have unpublished data on the size and weight of the placenta. The organs will be distributed to the investigators and if possible, to other investigators not involved (see attached)

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9. Physical Facilities Available :

The senior investigator has 400 square feet of laboratory space equipped with the following items.: 1 vibrationless steel optical bench for vital microscopy; quartz-rod apparatus; Leitz stereo binocular microscope modified for vital microscopy; Leitz Panphot microscope (without optics); RCA Vidicon television system; Fairchild/Dumont Vidicon television system; 8" Conrac TV monitor; Bolex H-16 Rex 5 16 mm. motion picture camera adapted for cinémicrophotography; 2-tripods; motion picture editing and storage equipment; YSI temperature control equipment; balances; Sage air curtain incubator; Heidbrink closed-circuit anesthetic machine; Wilnot Castle surgical lamp; Bausch and Lomb spectrophotometer; A. O. microtome; warming table; clinical centrifuge; A. O. microstar microscope; deionizer; microhematocrit centrifuge; IL 182 CO-oximeter.

Dr. Zimny has in her laboratory a A.E.I. - 6B transmission electron microscope; furthermore, she has available for her use a scanning electron microscope, J.S.M. - U3, at Touro Infirmary in New Orleans.

The Department of Anatomy also maintains adequate dark room and animal care facilities.

10. Additional Requirements:

None

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Thomas Hill Shepard

PUBLICATIONS

1. Shepard TH, Clauson SW: Case of adrenogenital syndrome with hypertension treated with cortisone. *Pediatr.* 8:805-813, Dec. 1951.
2. Wilkins L, Grumbach MM, Van Wyk JJ, Shepard TH, Papadatos C: Hermaphroditism: classification, diagnosis, selection of sex and treatment. *Pediatr.* 16:287-299, Sept. 1955.
3. Van Wyk JJ, Grumbach MM, Shepard TH, Wilkins L: The treatment of hyperthyroidism in childhood with thiouracil drugs. *Pediatr.* 17:221-229, Feb. 1956.
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5. Shepard TH: Carbonic anhydrase activity in blood of patients with chronic respiratory disease. *Amer. J. Med. Science* 233:162-166, Feb. 1957.
6. Thuline H, Shepard TH, Creighton SA: Chromosomal sex test: applications in pediatrics. *A.M.A. J. Dis. Child.* 94:130-136, Aug. 1957.
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10. Shepard TH, Landing BH, Mason DG: Familial Addison's disease case reports of two sisters with corticoid deficiency unassociated with hypoaldosteronism. *A.M.A. J. Dis. of Child.* 97:154-162, Feb. 1959.
11. Shepard TH, Creighton SA, Krebs EG, Lee LW, Thuline HG: Primary hyperoxaluria I. Clinical and pathologic findings in a patient with calcium oxalate nephrocalcinosis. *Pediatr.* 25:582-591, Apr. 1960.
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14. Shepard TH, Nielsen RL, Johnson ML, Bernstein N: Human growth hormone, I. Metabolic balance studies carried out in a hypopituitary child. *A.M.A. Dis. of Child.* 99:74-80, Jan. 1960.
15. Shepard TH, Gartler S: Increased incidence of non-tasters of phenylthiocarbamide among congenital athyreotic cretins. *Science* 131:929, March 1960.

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-26-

NAME: Marilyn L. Zimny

TITLE: Professor of Anatomy

BIRTHDATE: REDACTED

PLACE OF BIRTH: REDACTED

EDUCATION:

University of Illinois, Urbana, Illinois

Chemistry - major, Zoology - minor, B.A., 1948

Loyola University Stritch School of Medicine, Chicago, Illinois
Anatomy, M.S., 1951

Loyola University Stritch School of Medicine, Chicago, Illinois
Anatomy, Ph.D., 1954

PROFESSIONAL EXPERIENCE:

Professor - Anatomy, Louisiana State University Medical Center,
1964 - present

Associate Professor - Louisiana State University Medical Center,
1959 - 1964

Assistant Professor - Louisiana State University Medical Center,
1954 - 1959

Visiting Professor in Anatomy, University of Costa Rica, School
of Medicine, February-June, 1961 - 1962

Sabbatical leave, Institute of Arctic Biology, University of
Alaska, 1966

Abstractor for Biological Abstracts, 1959 to present
The World Book Encyclopedia Biology Committee

ORGANIZATIONS:

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incidences of lower birth weights or mortalities. Many of these reports strongly suggest a cause-and-effect relationship between CO and fetal development in mothers who smoke. The hypoxemia which occurs from exposure to CO is most often mentioned as the harmful effect elicited by CO per se, either from cigarette smoke or other sources. If the hypoxemia is truly responsible for the pre-natal or post-natal alterations from mothers exposed to CO, then the only way this hypoxic effect might be overcome would be by either increased production of hemoglobin by the mother, or an increased maternal blood flow to the placenta.

Experiments conducted in my laboratory during the past year, however, suggest that CO may have other effects upon the fetus in addition to its known effects upon the oxyhemoglobin dissociation curve. My experiments to date have shown that carbon monoxide administered to the mother at concentrations of 100-1000 p.p.m. in an air mixture will cause an increase in the maternal and fetal COHb level in rabbits comparable to or slightly higher than that of smokers (5-25% COHb). The exposure to CO in the mother causes a linear increase in her COHb levels throughout a four hour observation period. Fetal blood samples taken after completion of in vivo observations on the fetal mesentery revealed a similar or slightly higher per cent of saturation of hemoglobin by carbon monoxide. The oxyhemoglobin level (%) decreased in the adult concomitant with the rise in COHb levels. The hematocrit and total hemoglobin (g/100ml) did not change appreciably throughout the course of the experiments.

The response of the fetal microvascular system to increased levels of COHb is a vasoconstriction in the small arteries and veins (100-300 μ I.D.) of the mesentery followed by a progressive decrease in the linear velocity of blood flow through these vessels. These hemodynamic events preceded the eventual breakdown of the endothelial lining of the capillaries and

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described by these authors were found in large vessels and only non-cellular plasma constituents permeated the endothelium, their studies still support a direct toxic effect of CO on vascular endothelium. Astrup et al.¹³ further described an acceleration by CO alone in the development of atheromatosis of the aorta. They believed that the edematous condition and higher protein content of the aortic walls was due to an increased endothelial permeability. In the ultrastructural studies conducted by Kjeldsen et al.¹⁴ on the intimal changes in the rabbit aorta after moderate CO exposure, edema was evident under the basement membrane as well as the endothelial cells; often endothelium completely separated from the basement membrane and a plaque was formed. Of most interest in this study was the presence of tiny hemorrhages with platelet and red blood cell plugging in the areas of denuded endothelium. Kjeldsen et al.¹⁴ concluded that the morphologic intimal changes of the rabbit aorta were due to CO per se since the oxygen tension did not change during CO exposure. This was not the case in my experiments, since oxygen tension did decrease with a concomitant rise in COHb. Siggaard-Andersen et al.¹⁵ also reported that CO induces endothelial damage and that CO has a more pronounced effect than hypoxia alone on the permeability of capillaries to albumin. The exact mechanism by which CO increases endothelial permeability to plasma and/or cellular components of blood remains obscure; however, oxygen dependent enzymes may be necessary in order to maintain the permeability of individual endothelial cells and/or intercellular endothelial junctions. CO may in some way have a direct toxic effect upon these same enzymes.

The results of experiments on rabbit fetuses in my laboratory and those conducted by others on adult animals strongly suggest that CO can compromise the blood flow to tissues by causing a vasoconstriction of

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B. Significance:

The specific response of the fetal microvascular system to maternal exposure of carbon monoxide or nicotine has not been reported due, in part, to the difficulty in studying these vessels in vivo with the light microscope while maintaining homeostasis. In general, the response of the fetal microvascular system to any maternal agent is poorly understood due, in part, to the lack of information in man and animals regarding the transfer of substances across the placenta to the fetus.

During this past year, my laboratory has been conducting in vivo studies on the response of the fetal microvascular system to maternal carbon monoxide exposure. The main purpose of this study was to observe any changes in the dynamic structure or function of the fetal microvascular system which may occur after exposure of the mother to carbon monoxide in order to possibly explain the reported cause-and-effect relationship between maternal smoking and impairment of fetal growth and development. Since mothers who smoke have increased circulating carboxyhemoglobin (COHb) levels, possibly the carbon monoxide (CO) per se may have detrimental effects upon the fetus, particularly since CO is known to cross the placental barrier.¹ In this regard, Astrup et al.² found that mothers exposed to 180 p.p.m. of CO had a 20% decrease in birth weight and a neonatal mortality rate of 35%. They suggested that the CO content of cigarette smoke may be responsible for these two occurrences. In studies conducted by Meyer and Comstock,³ perinatal mortality increased if the mother had smoked during pregnancy. Several authors⁴⁻⁹ have suggested that the lower birth weights and increased mortality of babies born from mothers who smoke may be related to the relative hypoxemia in the fetus caused by the CO since babies born at high altitudes often have similar

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post-capillary venules resulting in the formation of petichial hemorrhages along the course of these vessels (capillaries and post-capillary venules) and widespread congestion within the capillary network of the fetus. The per cent of vasoconstriction (compared with control, before CO administration) in the fetus increased with time and with the level of maternal COHb. The observation period was never longer than four hours. Furthermore, the degree of extravasation of red blood cells from the capillaries or post-capillary venules and the amount of congestion within the microscopic field also increased with time and with the level of maternal COHb. The maximal response to the increased COHb levels was cessation of flow through terminal arterioles, capillaries, and post-capillary venules, with a great reduction in the linear velocity of blood flowing through the small arteries and veins in the mesentery. Due to the congestion within the capillary bed, the majority of the blood flowing in the small arteries would bypass the capillary network by flowing into arteriovenous anastomoses into small veins or venules. Control animals allowed to breathe room air or an air/gas mixture did not develop the microvascular lesions observed in fetuses exposed to CO for similar periods of time up to four hours.

Once a high level of maternal COHb was reached, the toxic effects of CO on the fetus were not reversible since removal of the CO stimulus after the initial vasoconstriction does not reverse the further effects upon endothelial permeability of capillaries and post-capillary venules. This is probably explained by the fact that the COHb levels, once elevated (20-25%), will not fall in time to prevent further damage to the endothelium of the capillaries and post-capillary venules. The results suggest, however, that if the CO stimulus is removed before the COHb levels reach 10% in the mother, only a slight vasoconstriction of small arteries and veins will be observed.

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PUBLICATIONS:

1. Zimny, M. L. and Rigamer, E. Glomerular ultrastructure in the kidney of a hibernating animal. *Anat. Rec.* 154: 87-94, 1966.
2. Zimny, M. L., Sherman, M. and Romano, C. C. Ultrastructural modifications of the intercalated disc during hypothermia in the rat and the ground squirrel. *Cryobiology* 4: 317-328, 1968.
3. Zimny, M. L. Glomerular ultrastructure in kidneys from some northern mammals. *Comp. Physiol. & Biochem.* 27: 859-864, 1968.
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5. Redler, I. and Zimny, M. L. Scanning electron microscopy of normal and abnormal articular cartilage and synovia. *Journal of Bone and Joint Surgery* 52A: 1395-1404, 1970.
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8. Booth, W. V., Zimny, M. L., Kaufman, H. J. and Cohn, I. Scanning electron microscopy of small bowel strangulation obstruction. *Amer. J. Surg.* 125: 129-133, 1973.
9. Zimny, M. L. and Redler, I. Variations in morphology of cartilage within a given area of articular surface. (Submitted for publication, *J. Microscopy*).

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11. & 12. Biographical Sketches of Professional Personnel and
Pertinent Publications:

Name: Samuel G. McClugage, Jr.

Birth Date: -

Birth Place: -

REDACTED

Education: Undergraduate: Millikin University, Decatur, Illinois
A.B. (Zoology), 1966.

Graduate: University of Cincinnati, College of
Medicine, Cincinnati, Ohio
Ph.D. (Anatomy), 1970.

Honors: N.I.H. Predoctoral Fellowship, 1967-1970

Consultant, Proctor and Gamble Company, Cincinnati, Ohio
1972 -

Recipient, Microcirculatory Society-Pharmacia Travel Award
(to visit research laboratories in Scandinavia), June, 1973.

Societies:

REDACTED

REDACTED

Research Interests: In vivo microscopy of living cells, tissues, and organs
in situ under normal or pathologic conditions; in vivo
physiologic and pharmacologic studies; microcirculation;
hematology; application of television and electronic
techniques to microscopic study of living tissues and
organs in situ.

- Background:
1. Assistant Professor of Anatomy, Louisiana State University Medical Center, 1971 - present
 2. Postdoctoral Fellow in Anatomy, University of Cincinnati, 1970-1971
 3. Pre-doctoral Fellow, National Institutes of Health (GM-38179), University of Cincinnati, 1967-1970
 4. Pre-doctoral Fellow, from the Dean of the College of Medicine, University of Cincinnati, 1966 - 1967
 5. Assistant Instructor in Biology, Millikin University, 1966

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Drs. Bing
Jacobson
Meier

Miscellaneous

#912

THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 59TH STREET
NEW YORK, N. Y. 10022

Application For Research Grant

MAY 29 1973

Date: May 16, 1973

1. Name of Investigator(s): (include Title and Degrees)

Samuel G. McClugage, Jr., Ph.D., Assistant Professor, Department of Anatomy
Marilyn L. Zimny, Ph.D., Professor, Department of Anatomy

2. Institution &

Address: Louisiana State University Medical Center
Department of Anatomy
1100 Florida Avenue
New Orleans, Louisiana 70119

3. Short Title of Project:

Microvascular Response of Fetus to Carbon Monoxide or Nicotine

4. Proposed Starting Date:

October 1, 1973

5. Anticipated Duration of this Specific Study:

October 1973 through September 1976

6. Brief Description of Objectives or Specific Aims:

(PLEASE SEE PAGE 2)

1003542278

7. Give a Brief Statement of your Working Hypothesis:

(SEE PAGE 3)

mild exposure to CO can induce pathologic changes in the walls of vessels in rabbits, it is conceivable that these changes could have an effect upon the flow of blood through these vessels. Thus, the experiments on the response of the adult microvascular system will be compared to: (1) the response of the fetus; (2) ultrastructural studies conducted by other authors in adult animals or man; and (3) the ultrastructural results of our own studies. If CO increases the permeability of endothelium to plasma constituents, then it may well be that CO does play a significant role in the development of coronary heart disease and even peripheral vascular diseases as has been suggested by several authors. If our scanning and transmission electron microscopic studies of fetal blood vessels which have been exposed to CO demonstrate a structural similarity to adult blood vessels that have been exposed to CO, then can one conclude that such morphologic changes might predispose a newborn animal (or human) to cardiovascular disease in later life? The dovetailing of information gathered from vital microscopic, scanning electron microscopic, and transmission electron microscopic studies of fetal vessels exposed to CO in this study may either support or refute this possibility.

The results from the CO experimental groups will be compared to the results from the nicotine experimental groups. Little information is available on the effects of nicotine on the fetus. The potentially harmful effects of nicotine on the fetus are just as important as those effects which may be related to CO. In fact, nicotine has been implicated more often than CO as the main harmful constituent of tobacco smoke due, in part, to its known pharmacological effects on the cardiovascular system. Nicotine is known to cross the placental barrier in some animals such as rats.¹⁶ In these animals, the fetal levels of nicotine actually exceeded the maternal levels at various intervals of time after maternal administration of radioactive nicotine. Nicotine is known to induce a significant

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Page 7

Thomas Hill Shepard

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62. Nelson T, Oakley GP, Shepard TH: A centralized laboratory for collection of human embryos and fetuses: Seven years experience: II classification and tabulation of conceptual wastage with observations on type of malformations, sex ratio and chromosome studies. Ibid.
63. Shepard TH, Smith DW: Prenatal life in Introduction to Clinical Pediatrics edited by D.W. Smith and R.E. Marshall. W.B. Saunders, Philadelphia, 1972.
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71. Robkin MA, Shepard TH: In vitro culture of somite-stage rat embryos: A new technique for maintaining growth and continuously monitoring heart rate. Applications in metabolic and teratologic studies. In Vitro 8: 151-160, 1972.
72. Norris JA, Robkin MA, Shepard TH, Tanimura T: Prompt effects of radiation on the heart rate of a mammalian embryo. Radiation Res. 52: 579-587, 1972.
73. Shepard TH: Anencephaly and potatoes. Letter to the editor. Lancet 1: 79, 1973.

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16. Other sources of financial support:

List financial support from all sources, including own institution, for this and related research projects.

CURRENTLY ACTIVE			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
	None		
PENDING OR PLANNED			
Title of Project	Source (give grant numbers)	Amount	Inclusive Dates
Lead and Cadmium Levels in Human Milk and Human Deciduous Teeth	(submitted to NSF, EPA)	\$37,222	1 Jan. '74 - 30 Dec. '75

It is understood that the investigator and institutional officers in applying for a grant have read and accept the Council's "Statement of Policy Containing Conditions and Terms Under Which Project Grants Are Made"

Checks payable to

Vanderbilt University

Mailing address for checks

c/o J. L. Weinberger

Vanderbilt University

102 Kirkland Hall

Nashville, Tennessee 37235

Principal investigator

Typed Name David J. Wilson

Signature David J. Wilson Date 12 July 1973

Telephone 615 322-2633 —
Area Code Number Extension

Responsible officer of institution

Typed Name James R. Surface

Title Executive Vice-Chancellor

Signature James R. Surface Date 7/35/73

Telephone 615 322-2508 —
Area Code Number Extension

1003542202

D. References:

1. Cole, P. V., Hawkins, L. H. and Roberts, D. Effects on the fetus of smoking during pregnancy. *J. Obstet. Gynecol.* 79: 782-787, 1972.
2. Astrup, P., Olsen, H. M., Trolle, D., and Kjeldsen, K. Effect of moderate CO exposure on fetal development. *Lancet* (#7789): 1220-1222, 1972.
3. Meyer, M. B., and Comstock, G. W. Maternal cigarette smoking and perinatal mortality. *Am. J. Epidemiol.* 96: 1-10, 1972.
4. Permutt, S., and Farhi, L. Tissue hypoxia and carbon monoxide. In: Effects of chronic exposure to low levels of CO on human health, behavior, and performance. National Academy of Engineering, Washington, D. C., pp. 18-24, 1969.
5. Longo, L. D. Carbon monoxide in the pregnant mother and fetus and its exchange across the placenta. *Ann. N. Y. Acad. Sci.* 174: 313-341, 1970.
6. Hadden, W., Jr., Nesbitt, R. E. L., and Garcia, R. Smoking and pregnancy: CO in blood during gestation and at term. *Obstet. Gynecol.* 18: 262-267, 1961.
7. Lichty, J. A., Ting, R. Y., Bruns, P. D., *et al.* Studies of babies born at high altitude. *Am. J. Dis. Child.* 93: 666, 1957.
8. McClung, J. Effects of high altitude on human birth, observations on mothers, placentas, and the newborn in two Peruvian populations. Cambridge, Mass., Harvard University Press, 1969.
9. Grahn, D., and Kratchman, J. Variation in neonatal death rate and birth weight in the U.S. and possible relations to environmental radiation, geology, and altitude. *Am. J. Hum. Genet.* 15: 329-352, 1963.
10. McCuskey, R. S., McClugage, S. G., Moore, T. J., and Miller, M. L. Response of the fetal mesenteric microvascular system to maternal hypoxia. *Proc. Soc. Exp. Biol. Med.* 132: 636-639, 1969.
11. Brinkman, C. R., Weston, P., Kirshbaum, T. H., and Assali, N. S. Effects of maternal hypoxia on fetal cardiovascular hemodynamics. *Am. J. Obstet. Gynecol.* 108: 288-301, 1970.
12. Astrup, Poul. Some physiological and pathological effects of moderate CO exposure. *Brit. Med. J.* 25: 447-452, 1972.

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13. Justification of Budget:

A. Personnel

The personnel that will be required on this project are one full-time research assistant for Dr. McClugage and a half-time research assistant who will work with Dr. Zimny in the preparation of tissues for scanning and transmission electron microscopy. The percent used for calculating fringe benefits at Louisiana State University is 10% which has been included in the "amount column" of the budget.

B. Use of Scanning Electron Microscopy:

The Department of Anatomy at Louisiana State University Medical Center does not have a scanning electron microscope (SEM). However, we have an agreement with the Research Institute at Touro Infirmary in New Orleans to rent their SEM at a rate of \$20.00/hour. Dr. Zimny has access to this microscope whenever its use is needed.

C. Machinist Expenses:

The employment of a machinist who can make the necessary animal trays for use on the Panphot microscope, adapted for vital microscopy, will be necessary. These trays must meet certain specifications depending on the type of animal used and the particular organ which is to be observed in vivo.

D. Permanent Equipment :

1. Monochromatic System Adapted for Quartz Rod

Schoeffel Instrument Corporation recently manufactured a monochromatic system which provides maximum light energy (from 200-700nm.) with high spectral purity. The complete system consists of a Xenon or Xenon-Mercury arc lamp, power supply, arc lamp housing, double monochromators, and appropriate optics. The double monochromators provide a narrow spectral

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Council for Tobacco Research

CURRICULUM VITAE

Louis A. Vontver, M.D., M. Ed.PERSONAL DATA

Citizenship

REDACTED

Date of Birth

Place of Birth

REDACTED

Address

REDACTED

Marital Status

REDACTED

EDUCATION

High School

Powell High School
Powell, Wyoming

1949-53

College

(B.A.) Magna Cum Laude
University of Minnesota
Minneapolis, Minnesota

1953-56

(B.S.) Univ. of Minnesota
Minneapolis, Minnesota

1956-60

Medical School

(M.D.) Univ. of Minnesota
School of Medicine
Minneapolis, Minnesota

1956-60

Internship

Harbor General Hospital
Torrance, California

1960-61

Residency

Department of Obstetrics and
Gynecology
University Hospital
Seattle, WashingtonJuly 1965 -
June 1969

Chief Resident

Department of Obstetrics and
Gynecology
University Hospital
Seattle, WashingtonApril 1968 -
March 1969

Graduate Study

(M.Ed.) Univ. of Washington
Seattle, WashingtonApril 1969 -
December 1970

1003542272

MILITARY SERVICE

Captain	U.S. Air Force Reserves	
Active Duty	Tachikawa Air Force Base Japan	1961-64
Active Reserves	Paine Air Force Base Washington	1964-65

RESEARCH APPOINTMENTS

Fellowship	Department of Obstetrics and Endocrinology University Hospital Seattle, Washington	1964-65
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FACULTY POSITIONS

Instructor	Department of Obstetrics and Gynecology University of Washington School of Medicine	July 1969 - June 1971
Assistant Professor	Department of Obstetrics and Gynecology University of Washington School of Medicine	July 1971 - present

HOSPITAL POSITIONS

Consulting Staff at U.S. Public Health Service Hospital.
 Attending Staff at University Hospital and Harborview Medical Center...
 Assistant Chief, Harborview Medical Center 1972 - Present

MEDICAL LICENSURE

Minnesota (July 15, 1960)
 California (November 29, 1961)
 Washington (December 15, 1964)

BOARD CERTIFICATION

American Board of Obstetrics and Gynecology
 November 12, 1971

PROFESSIONAL ORGANIZATIONSFRATERNITIES

1003542273

REDACTED

REDACTED

camera. The use of monochromatic light used in conjunction with a black and white television system greatly improves the visualization of living tissues or organs since the contrast is greatly increased.

The 16 mm. motion picture camera may also be used for direct cinéphotomicrography. Throughout the in vivo experiments, the results are permanently documented for later reference. These results can be studied repeatedly and critically analyzed frame by frame in order to compare the sequential responses of the microvasculature in one animal to that of other animals. Thus, using this in vivo technique, the rate, duration, magnitude and direction of the response in the fetus or adult animal can be examined and recorded.

In one group of experimental animals, studies will be continued on the response of the fetal microvascular system to the maternal exposure of CO. To study the acute response of the fetal microvasculature to CO, the mother will receive a mixture of CO and air in varying concentrations of CO from .01% to .1% balance/air (100-1000 p.p.m.) using a closed circuit anesthetic machine. This range of CO will cause an increase in the COHb level of the female adult rabbit from 5 - 15% which mimicks COHb levels which have been reported in human studies on mothers who smoke. The fetal microcirculation will then be studied under the following experimental conditions: (1) after maternal anesthesia but before CO exposure, i.e., while the mother is breathing room air or air via the closed circuit anesthetic machine; (2) during maternal CO exposure; (3) during recovery when the mother is again allowed to breath room air. It should be emphasized that each pregnant animal can be used for each of the above experimental (CO) groups. Thus, each animal can be used as its own "control". The response of the fetal microvascular

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labeled dextran within the microvascular system can be examined in vivo by transilluminating the adult or fetal mesenteries with monochromatic light at a wavelength of 487 mμ and by using a Leitz barrier filter of 515 or 530. Alternatively, selective exciter filters (Leitz BG 12 or KP500) may be used in conjunction with proper barrier filters. Thus, the permeability of the microvascular system to the plasma containing dextran particles can be studied microscopically before and after exposure of the animal to CO. This, then, can be correlated with any increased permeability of cellular elements in the fetal or adult microvascular system. The dextran infusions can be used in any of the experimental groups using rabbits; however, it cannot be used in those groups using rats since rats are hypersensitive to dextran.

I have included a brief description of the technique in this letter in the hope that it may appended to the "methodology" section of the research grant since this technique can document in vivo the passage of plasma across the endothelium of the microvascular system. Furthermore, this technique is far superior to other plasma labeling techniques such as Evan's blue which have been employed in the past to study permeability of vascular endothelium. This in vivo technique will permit a more accurate description of the permeability of small vessels to plasma and will provide a better means to compare the results of this study to those conducted by others who have also similarly described effects of CO on endothelial permeability using other techniques such as electron microscopy.

This technique will not require any additional expenses in the budget. Furthermore, Pharmacia has provided me with a quantity of fluorescein conjugated dextran for use in my laboratory.

Thank you.

Sincerely,



Sam G. McClugage, Jr., Ph.D.
Assistant Professor

SGMc:sar

Enclosure

1003542311

EDUCATIONAL COMMITTEES OF UNIVERSITY OF WASHINGTON MEDICAL SCHOOL

- | | | |
|----|---|-----------|
| 1. | Human Biology 450 (Human Reproduction) | |
| | Member | 1969-70 |
| | Chairman | 1970-72 |
| 2. | Test and Evaluation Committee | 1969-70 |
| 3. | Curriculum Committee | 1970-1972 |
| 4. | Learning Resources Advisory Committee | 1971-72 |
| 5. | Ad Hoc Advisory Committee on Learning
Resources in Health Sciences | 1971-72 |

BIBLIOGRAPHY

Study Aid for Human Biology 450

1003542274

group of animals will provide information on another species of animal which can then be compared to the response of the microvascular system in adult rabbits. Furthermore, the use of a microscope (Leitz Panphot) which permits higher magnifications (100-1200X) than a stereo-binocular microscope may provide information which would not be obtainable with lower magnifications and poorer resolutions. Also, the microvascular system of liver is morphologically and functionally different from that of the mesentery; thus, the sensitivity of the two to CO or nicotine may also be quite different since one represents a microvascular bed in a relatively non-metabolic tissue (mesentery) versus one which is highly metabolic (liver).

During the experiments, the maternal and fetal hematocrit, hemoglobin concentration (g/100ml), oxyhemoglobin concentration (%) and carboxyhemoglobin (%) will be monitored, the latter three by an IL CO-Oximeter, in order to compare the response in the fetal or adult microvascular systems with any fluctuations in maternal or fetal blood parameters.

In each of the various experimental groups, samples of blood vessels will be taken after in vivo observations. These samples of blood vessels will be fixed in 2% gluteraldehyde, buffered with cacodylate, pH 7.4 for 24 hours, rinsed three times with buffer and stored in a refrigerator. Part of the sample will then be osmicated, dehydrated in graded alcohols, processed through amyl acetate and critical point dried. After drying, the tissue will be coated with carbon and gold palladium alloy and viewed in a JSM-U3 scanning electron microscope. The usual accelerating voltage used by the investigator in past studies of other tissues has been 15 KV. Observations of the tissue in question will be made at this accelerating voltage and other magnitudes of voltage will be tested so as to obtain the maximum visual results.

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system to CO will be compared to that of the adult microvascular system in order to compare the sensitivity of the fetus with that of the mother.

In a second group of experimental animals, the effects of nicotine on the fetal microcirculation will be examined after the administration of a subcutaneous dose to the mother. The dose to be used in rabbits will attempt to mimic that amount of nicotine absorbed by human smokers, which has been reported to be 1.0 to 2.0 mg. of nicotine per kilogram from a pack of cigarettes per day. As in the CO experimental group, each fetal preparation will be examined before, during, and after the maternal administration of nicotine. The response in the fetus will also be compared to that of the adult microvascular system.

After completion of experiments on the effects of CO or nicotine on the fetal microvascular system, the results from the two experiments will be compared for any similarities or differences in responses under the two experimental conditions. A third group of experiments which would be most interesting to perform would be to study the response of the fetal microvascular system during the maternal inhalation of cigarette smoke. However, to date, I am not aware of any mechanical device for exposing rabbits to cigarette smoke under conditions which simulate human exposure. There are, of course, means by which the effects of cigarette smoke could be examined in rabbits, but I question the value of these studies if they do not at least simulate conditions during human smoking. If such a device for rabbits becomes available during the course of the experiments, it could be easily incorporated into the experimental design of this study. I know that the Council for Tobacco Research is sponsoring work to develop mechanical devices for animals which simulate human conditions; thus, they are in a position to know when such a device for use with rabbits becomes available. The in vivo model which is to be used in the

1003542284

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14. Kjeldsen, K., Astrup, P., and Wanstrup, J. Ultrastructural intimal changes in the rabbit aorta after a moderate carbon monoxide exposure. *Atherosclero.* 16: 67-82, 1972.
15. Siggaard-Andersen, J., Petersen, F. B., Hansen, T. I., and Mellempgaard, K. Vascular permeability and plasma volume changes during hypoxia and CO exposure. *Angiol.* 20: 356-358, 1969.
16. Mosier, H. D., and Jansons, R. A. Distribution and fate of nicotine in the rat fetus. *Teratol.* 6: 303-312, 1972.
17. Westfall, T. C., and Watts, D. T. Effects of cigarette smoking on epinephrine secretion in the dog. *Proc. Soc. Exp. Biol. Med.* 112: 843-847, 1963.
18. Schievelbein, H., and Eberhardt, R. Cardiovascular actions of nicotine and smoking. *J. Natl. Canc. Inst.* 48: 1784-1794, 1972.
19. Becker, R. F., and Martin, J. C. Vital effects of chronic nicotine absorption and chronic hypoxic stress during pregnancy and the nursing period. *Am. J. Obstet. Gynecol.* 110: 522-533, 1971.
20. Matsubara, T., and Sano, T. Effect of cigarette smoking on human precapillary sphincters. *Br. J. Pharmacol.* 45: 13-20, 1972.

1003542299

CURRICULUM VITAE

Felix G. Freund, M.D.

Personal Data:**REDACTED**Education:

1938-41	Universidad Nacional de Buenos Aires	M.D.
1943	Medical School	
1946-48		

Postgraduate Training:

1949-50	Hospital Fiorito, Buenos Aires	Internship
1950-52	Hospital Florito, Buenos Aires	Residency Internal Medicine
1952-54	Rawson Hospital, Buenos Aires	Residency Anesthesiology
1954-56	Massachusetts General Hospital, Boston	Residency Anesthesiology

Faculty Positions Held:

1957-61	Instructor in Anesthesiology, Washington University, St. Louis, Missouri
1961-63	Assistant Professor of Anesthesiology, Washington University, St. Louis, Missouri
1963-65	Instructor, Department of Anesthesiology, University of Washington School of Medicine, Seattle, Washington
1965-70	Assistant Professor, Department of Anesthesiology, University of Washington School of Medicine, Seattle, Washington
1970-	Associate Professor, Department of Anesthesiology, University of Washington School of Medicine, Seattle, Washington

Hospital Positions Held:

1950-53	Junior Member, Internal Medicine Department, Hospital Fiorito, Buenos Aires
1957-63	Assistant Anesthetist, Barnes Hospital and Children's Hospital, St. Louis Missouri
1963-	Attending staff, Department of Anesthesiology, University of Washington Medical Center and Affiliated Hospitals, Seattle

Administrative Positions Held:

1970-	Director of Clinical Services, Department of Anesthesiology, Harborview Medical Center, Seattle
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Military Service:

1942	Armed Forces of Argentina (Cavalry)
1944-45	

1003542265

Page 6

Thomas Hill Shepard

46. Shepard TH: Development of the thyroid gland. Pp. 200-205 in Endocrine and Genetic Diseases of Childhood edited by L.I. Gardner, W.B. Saunders, Philadelphia, 1969.
47. Shepard TH, Fry LR, Moffet BC: Microscopic studies of achondroplastic rabbit cartilage. *Teratology* 2:13-22, Feb. 1969.
48. Shepard TH, Tanimura T, Robkin M: *In vitro* study of rat embryos. I. Effects of decreased oxygen on embryonic heart rate. *Teratology* 2:107-110, May 1969.
49. Shepard TH, Bass GL: Organ culture of limb buds from riboflavin-deficient and normal rat embryos in normal and riboflavin-deficient media. *Teratology* 3:165-167, May 1970.
50. Streissguth AP, VanderVeer BB, Shepard TH: Mental development of children with congenital rubella syndrome. *Am.J.Ob.&Gyn.* 108:391-399, Oct. 1970.
51. Tanimura T, Shepard TH: Glucose metabolism by rat embryos *in vitro*. *Proc. Soc.Exp.Biol.&Med.* 135:51-54, Oct. 1970.
52. Davis SD, Nelson T, Shepard TH: Teratogenicity of B₆ deficiency omphalocele, skeletal and neural defects, and splenic hypoplasia. *Science* 169:1329-1330, Sept. 1970.
53. Hellstrom I, Hellstrom KE, Shepard TH: Cell-mediated immunity against antigens common to human colonic carcinomas and fetal gut epithelium. *Int.J.Cancer* 6:346-351, 1970.
54. Shepard TH, Tanimura T, Robkin M: *In vitro* studies for analysis of teratogenic events in the Symposium on Congenital Malformations of Mammalia. Masson & Cie, Paris, 1971.
55. Shepard TH: Development of the human fetal thyroid. Pp. 767-780 in *Hormones in Development* edited by Max Hamburgh and E.J.W. Barrington, Chapter 60, Appleton-Century-Crofts, New York, 1971.
56. Shepard TH: Organ-culture studies of achondroplastic rabbit cartilage: Evidence for a metabolic defect in glucose utilization. *J.Embryol.&Exp.Morph.* 25: 347-363, June, 1971.
57. Green HG, Gareis FJ, Shepard TH, Kelley VC: Cretinism associated with maternal sodium iodide I 131 therapy during pregnancy. *Amer.J.Dis.Child.* 22:247-249, Sept. 1971.
58. Tanimura T, Nelson T, Hollingsworth RR, Shepard TH: Weight standards for organs from early human fetuses. *Anat. Rec.* 171:227, 1971.
59. Shepard TH, Tanimura T, Robkin MA: Energy metabolism in early mammalian embryos. *Dev. Biol. Supp.* 4: 42, 1970.
60. Bleyer WA, Hakami N, Shepard TH: Development of hemostasis in the human fetus and newborn infant. *J. Pediat.* 79:838, 1971.

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various experimental groups would be a means for examining the effects of maternal smoking on the response of the fetal microvascular system, and then to compare this response with the nicotine-treated and CO-treated animals.

One further experimental group of animals will be used to study in adult female Sprague-Dawley rats (100-125g) the response of the mesenteric and hepatic microvascular system to carbon monoxide or nicotine. Animals will be anesthetized by intraperitoneal injection of urethane (ethyl carbamate). To expose the liver or mesentery of the rat, a midline and subcostal incision will be made and the liver or mesentery exteriorized by floating it onto a window of Saran Wrap which overlays a substage condenser of a Leitz Panphot microscope. Homeostasis will be maintained by irrigating the field with physiological Ringer's solution kept at the body temperature of the animal by heat regulators. Transillumination of the tissue will be accomplished by using a technique modified after Bloch and Coyas (Anat. Rec. 145: 374, 1963). With the liver or mesentery in position over the substage condenser, microscopy of the tissue is accomplished by passing a beam of monochromatic light through the condenser of a modified Leitz Panphot microscope. As mentioned earlier, the use of monochromatic light aids in the visualization of tissues and when used in conjunction with a black and white television system, the contrast can be greatly improved. The transilluminated tissues can then be observed by direct microscopy at magnifications of 100-1200X using Leitz water immersion or U.M.K. objectives with appropriate oculars or the optical image can be projected onto the photocathode of a television system.

The adult rats will be exposed to CO and nicotine in a similar manner as described for rabbits before, during, and after exposure. This experimental

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It is interesting to compare the results of these experiments with those experiments performed in the past on the response of the fetal mesenteric microvascular system to maternal hypoxia¹⁰. In this earlier study, maternal hypoxia induced a vasoconstriction in the fetal microvascular bed which seemed to be mediated by an oxygen dependent alpha-adrenergic mechanism since recovery from this vasoconstriction coincided with the return of the pO_2 to normal values after removal of the hypoxic stimulus to the mother. In these experiments, this recovery occurred within 20 minutes.

The results of my experiments suggest that the vasoconstriction in the fetal microvascular system may be due to fetal hypoxemia which occurs with increased levels of COHb since hypoxia alone, induced by a low oxygen gas mixture, produced a similar vasoconstriction. Other authors¹¹ have also reported increased fetal systemic vascular resistance during hypoxia in pregnant ewes, although one cannot assume that an increased fetal systemic vascular resistance reflects what is occurring in a particular microvascular bed of an animal.

It is more difficult to explain the endothelial damage induced by CO per se or hypoxia. In this regard, the extravasation of red blood cells through the endothelium represents some type of endothelial damage. This increased permeability of endothelium after exposure to CO has also been described by other authors in "adult" animals or human studies. Astrup¹² found that cholesterol-fed adult rabbits exposed to CO had a greater accumulation of cholesterol in their arterial walls (aorta) when compared to only cholesterol-fed controls. Furthermore, Astrup and his associates found that CO (9-10% COHb) alone induces arterial lesions hallmarked by subendothelial edema indistinguishable from the intimal appearance of spontaneous arteriosclerosis. Even though the lesions

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Other Sources of Financial Support

List financial support for research from all sources, including own institution, for this and/or related research projects.

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Title of Project	Source	Amount	Duration
Response of the fetal mesenteric microvascular system to maternal carbon monoxide exposure	Louisiana Heart Association	7,300.00	July, 1972- June, 1973
<u>In vivo</u> model for testing effects of pulp capping agents on dental pulp	Institutional Grant	900.00	March, 1973- February, 1974

ending

(None)

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will be conducted on the response of the mesenteric microvascular system in rats to CO or nicotine and compared to the response in "adult" rabbits. This will permit a comparison of the sensitivities to CO or nicotine of these two animals in the same microvascular bed. Furthermore, in order to study the effects of CO or nicotine on another microvascular bed, in vivo microscopic studies will be conducted in rats on the response of the hepatic microvascular system to CO or nicotine; these results will then be compared to the response in the mesentery. This will provide useful information on the sensitivities of different microvascular beds in the same animal (hepatic and mesenteric in rats) versus similar microvascular beds in two different animals (mesenteric in adult rabbits and rats).

After completion of the acute experiments outlined in this proposal, hopefully additional information will be available which either supports or refutes (a) the reported cause-and-effect relationship between cigarette smoking and fetal or neonatal development, and (b) the etiologic role cigarette smoking plays in the development of various cardiovascular diseases in adults. By examining the separate effects of CO and nicotine on the adult and fetal microvascular systems in animals, one may gain a better insight into the problem of defining what constituents of cigarette smoke are truly harmful.

C. Addendum:

In the experimental protocol, one potential experimental group was the effects of maternal smoking on the fetal or adult microvascular system. These studies depended upon the availability of a mechanical device which would simulate human exposure to cigarette smoke. I would like to re-emphasize that if such a machine becomes available, this experimental group (exposure to smoke) will be added. I believe that this would be an integral part of the proposal since one could observe in the living animal if the CO -

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bandwidth at the selected wavelengths while suppressing stray light at other wavelengths to 1 part in 100,000. This system has a focusing sleeve at the exit portal which would permit the beam being focused on the quartz rod which has been used to date for transillumination using only white light. The main problem with white light is the inability to selectively build-up the contrast of the optical system. The use of monochromatic light permits the selection of wavelengths of light that are absorbed by specific tissue and cellular components. This differential absorption of light by these structures enhances their contrast with the surrounding structures and aids in their visual recognition. When such differences of absorption are sensed by the television tube and converted into an electronic image, the contrast between tissue and cellular components can be enhanced further by adjustment of the brightness and contrast controls on the video monitor. For example, patterns of blood flow can be followed more easily than by using white light by selecting a wavelength of light that is absorbed maximally by hemoglobin in red blood cells (414 mμ). This system will allow more critical observations of the linear velocity of blood flow through the microvascular system as well as passage of these cells through the endothelium of these vessels.

2. Optical Equipment for Panphot Microscope:

At the present time in my laboratory, a fused quartz rod is being used as a light source. The use of a quartz rod (coupled with a monochromatic system) as a transilluminatory light source can provide an adequate amount of light for transillumination of thin tissues such as the fetal or adult mesentery in the rabbit. However, the investigator is somewhat limited in respect to the tissues or organs selected for study since relatively low magnifications are used. Thicker tissues or organs require higher intensities of light in order to

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THE COUNCIL FOR TOBACCO RESEARCH - U.S.A., INC.

110 EAST 58TH STREET
NEW YORK, N. Y. 10022

Application For Renewal of Research Grant

JUL 31 1973

First ☐ Second ☐ Third ☒

Date: July 27, 1973

1. Name of Investigator(s): (include title and degrees)
Gary D. Friedman, M.D., M.S., Principal Investigator
and Carl C. Seltzer, Ph.D., Co-Investigator

2. Institution & Address:
Department of Medical Methods Research
Kaiser Foundation Research Institute
3779 Piedmont Avenue
Oakland, California 94611

3. Short Title of Project:
Characteristics of Smokers and Non-Smokers

4. Proposed Renewal Starting Date: (Anniversary or other) February 1, 1974

5. Discuss any Important Changes or Additions to Objectives or Specific Aims:

Please see attached Progress Report #3 for a brief review of our accomplishments to date.

Our objectives continue to be a thorough study of the characteristics of smokers as compared to non-smokers. During the proposed renewal year we would like to place more emphasis on areas in our data that we have not concentrated on in the past. These would include such items as developmental characteristics (e.g., age at menarche, age at menopause) family history, and medical history questions. Also, while we have made a few analyses of changes in characteristics according to changes in smoking status (in our papers about serum chemistry tests and about the leukocyte count) we would like to put considerably more effort into this area. We believe that this longitudinal look at the data will reveal relationships more clearly than can be found in the cross-sectional analyses that we have been carrying out. In order to increase the time span covered we plan, for selected variables, to add more recent data to the 1964-1968 period on which we have been focussing.

6. Give a Brief Statement of your Working Hypothesis if altered or modified:
No change

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Equieffective pressor doses of nicotine, acetaldehyde and tyramine will be compared in animals after the surgical or pharmacologic procedures described above. Data will be expressed as percent change from resting blood pressure and heart rate. Drugs will be administered in randomized fashion and experiments terminated if resting parameters do not return to initial pretreatment levels. Statistical comparisons will be made according to methods described in Steel and Torrie (Principles and Procedures of Statistics: McGraw-Hill Book Co., New York, 1960).

In vivo experiments will be carried out during the first year of the project. The experiments indicated can be separated into three blocks: (1) Dose-response relationships between intravenous nicotine, acetaldehyde and tyramine; (2) Investigation of alterations of these responses by surgical and pharmacologic procedures; and (3) An investigation of interactions between the three indirectly acting sympathomimetic agents.

In vitro experiments: (1) Central ear artery. Rabbits weighing 2-3 kg will be anesthetized with pentobarbital sodium (30 mg/kg) administered intravenously. The central ear artery will be cannulated in situ and removed according to the method described by de la Land and Rand (Aust. J. Exp. Biol. Med. Sci. 43:639, 1965). The prepared artery will be perfused with Krebs-bicarbonate solution in an organ bath kept at 37°C with a rate of 4-6 ml/min. from a constant volume pump. Drugs will be administered as closely as possible to the artery. Responses will be recorded on a Grass polygraph.

Arteries will be equilibrated for 30 minutes before exposure to sympathomimetic agents. Both the perfusion solution and solution in the organ bath will be aerated with a mixture of 95% oxygen and 5% carbon dioxide. Drug injections will be made either into the perfusion solution or tissue bath to assess intra- and extraluminal effects.

The concentration of ionic calcium in Krebs-bicarbonate solution is 2.4 mM. Ionic calcium concentration in solution will be increased from calcium-free up to three times normal. Each time the solution perfusing the artery and in the organ bath is changed, 30 minutes will be allowed for equilibration before sympathomimetic agents are added. Transmural stimulation of the isolated ear artery will be achieved via platinum electrodes and a Grass model S48 stimulator. Preliminary experiments are underway to establish the voltage, frequency and duration required to duplicate responses elicited by drug injection.

(2) Rat vas deferens. Male Wistar rats will be sacrificed by cervical dislocation. The vas deferens will be removed and carefully dissected free of vascular tissue and suspended vertically in a tissue bath containing 40 ml of Tyrode solution. A resting tension of one gram will be placed on each vas deferens and tissues allowed to equilibrate for 15 minutes before submaximal voltage responses are determined.

Transmural stimulation will be performed according to Brimingham and Wilson (Brit. J. Pharmacol. 21:569, 1963) with a pair of platinum electrodes. One electrode is inserted into the lumen of the prostate end of the vas deferens and the other electrode is positioned in the tissue bath. Stimulation is applied for periods of 5 seconds every 2 minutes at 25 Hz and a pulse width of one second.

After constant responses are recorded, guanethidine (4×10^{-6} M) will be added to the tissue bath. Thirty minutes later when the contractile responses are abolished, antagonists will be added to the tissue bath without changing the bath solution and one in which the bath solution is changed once. Contractile responses will be recorded on a Grass polygraph by means of force displacement transducers. Data will be expressed as percent of the original responses of vas deferens to transmural stimulation before the addition of guanethidine. Duncan's multiple range test will be used to identify significant differences among ranked means (Steel and Torrie, 1960).

Tissue content of guanethidine will be determined at the end of each experiment in order to determine whether or not the sympathomimetic agents were able to reduce tissue binding. Vas deferens will be removed from the tissue bath, washed three times, blotted and weighed. Tissues will then be homogenized in 0.01 N HCl and the homogenate extracted with CHCl_3 for assay of guanethidine according to the method of Chang et al (J. Pharmacol. Exp. Ther. 147:303, 1965).

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Following scanning electron microscopic observations, the sample may be placed in propylene oxide and embedded in plastic for further study with the transmission electron microscope. The plastic embedment material used in our laboratory is either Maraglas or Araldite. After polymerization of the plastic embedment, 1 μ plastic sections will be stained with Paragon and viewed with a light microscope for purposes of orientation. Ultra thin sections will then be made, stained with uranyl acetate and lead citrate and viewed with an A.E.I.-6B transmission electron microscope.

In addition to viewing the same piece of tissue with both scanning and transmission electron microscopy, it will also be possible to use part of the original sample that was fixed in 2% gluteraldehyde, buffered with cacodylate, pH 7.4, solely for transmission electron microscopic observation. For this, part of the original fixed sample would be osmicated, dehydrated in graded alcohols, placed in propylene oxide and subsequently be embedded in Maraglas. Once again thick plastic sections would be stained with Paragon and the following ultra thin sections would be stained with uranyl acetate and lead citrate and viewed in a transmission electron microscope.

If deemed necessary, for correlation with scanning or transmission electron microscopic observations, part of the originally fixed sample can also be prepared for light microscopy. For this purpose part of the original fixed sample would be dehydrated in graded alcohols and embedded in paraffin. Paraffin sections could then be stained for routine histological observations or stained with special chemicals so as to visualize various fibrous components of the tissue or possible lipid inclusions.

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small arteries and veins and by increasing the endothelial permeability to plasma and/or cellular constituents of blood. These functional or morphological alterations can severely compromise the perfusion of capillaries thus impairing the proper delivery of oxygen to tissues or organs. This, then, would be an additional effect of CO upon the already compromised oxygenation of the blood due to formation of the inactive pigment, carboxyhemoglobin.

The results from these studies lend further support to the possibility that the CO content of cigarette smoke may be the causative agent which is responsible for the lower birth weights of newborn or the higher incidence of neonatal mortality in newborns from mothers who smoke. The microvascular effects described in this study coupled with the known effects of CO on oxygenation could impair the proper delivery of blood to the growing fetus. The functional and morphologic alterations which may arise in the fetus, then, really only depends upon whether or not the microvascular response observed in the fetal mesentery is truly representative of what occurs in other microvascular beds such as the central nervous system.

The experimental protocol in this proposal will further investigate the effects of CO on the fetal microvascular system. The use of monochromatic light will provide additional information on the alterations in structure and function of the microvascular system induced by CO by enabling more critical observations of the microvascular response at one wavelength of light, for example, 414 mμ for hemoglobin. Since studies will also be conducted on the maternal microvascular response to CO, it will be interesting to ascertain if this response mimicks that in the fetus. Since several of the ultrastructural studies by Kjeldsen et al.¹⁴ and others suggest that a

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increase in the amount of catecholamines released from the adrenal gland.¹⁷ If nicotine does cross the placental barrier in pregnant rabbits and if it does increase circulating levels of catecholamines due to its action on the fetal adrenal gland, then the peripheral vascular resistance and/or cardiac output might be markedly affected. Nicotine is also known to have other effects such as accelerating platelet aggregation by ADP.¹⁸ Like CO, nicotine also has been shown to affect the birth weight and neonatal or prenatal mortality rate of offspring of mothers who received nicotine.¹⁹ The vasoconstriction and breakdown of the endothelial lining of the fetal microvascular system after CO may also occur with nicotine since Matsubara and Sano²⁰ suggested that nicotine induces closure of pre-capillary sphincters in calves causing a decreased capillary filtration coefficient. Although the studies by Matsubara and Sano²⁰ were performed in calves, other authors have described the effects of nicotine on the fetus and have suggested that the response depends upon the gestational age of the fetus which, in turn, reflects the development of the autonomic nervous system and adrenal gland. Thus, nicotine can play a similar role as CO in compromising the blood flow and/or oxygenation of growing fetuses; thus it is put in a similar category as a potentially harmful etiologic agent of tobacco smoke. It will be interesting to compare the response of the fetal microvascular system to the exogenous administration of nicotine to the mother with the response of maternal carbon monoxide exposure. Then one may be able to better appreciate the mechanisms which function in the fetus to produce deleterious effects upon fetal growth and development in mothers who smoke.

As mentioned in the experimental protocol, the studies conducted on the response of the "adult" microvascular system in rabbits after exposure to CO or nicotine will be repeated in adult rats. Thus, in vivo observations

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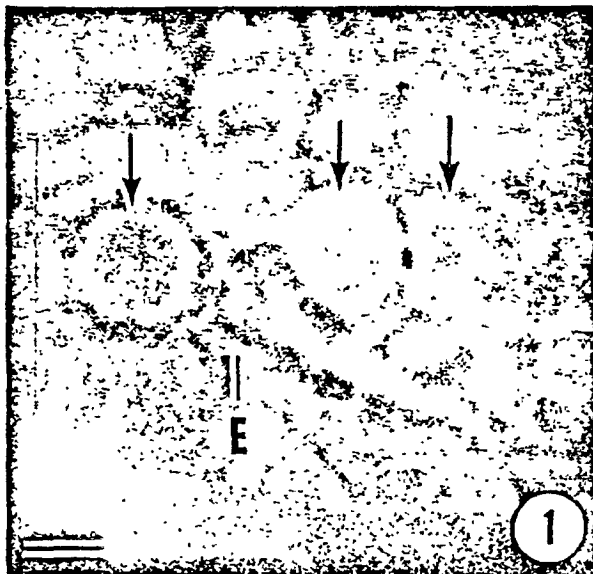


FIG. 1. Aggregation and adhesion of white blood cells (arrows) to endothelium (E) of sinusoid 2 hr after administration of CCl_4 . Single frame from motion picture. Size marker is 5μ .

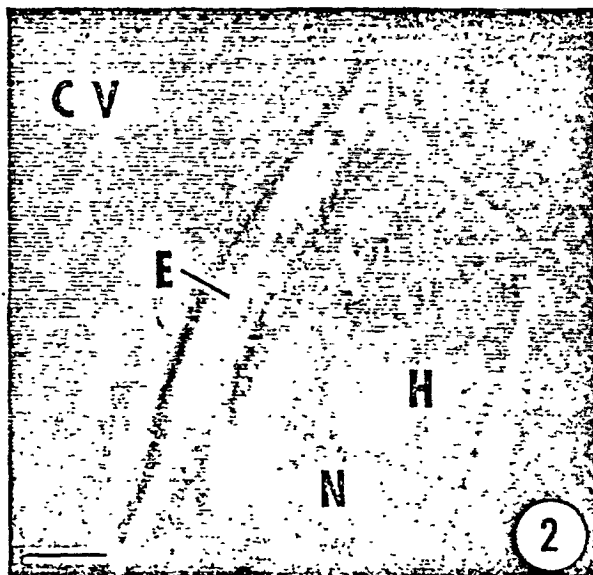


FIG. 2. Central venule (CV) with optimal circulation in nontreated, healthy liver. Note that there are no white blood cells adhering to the endothelium (E); N, nucleus of hepatic cell (H). Single frame from motion picture. Size marker is 5μ .

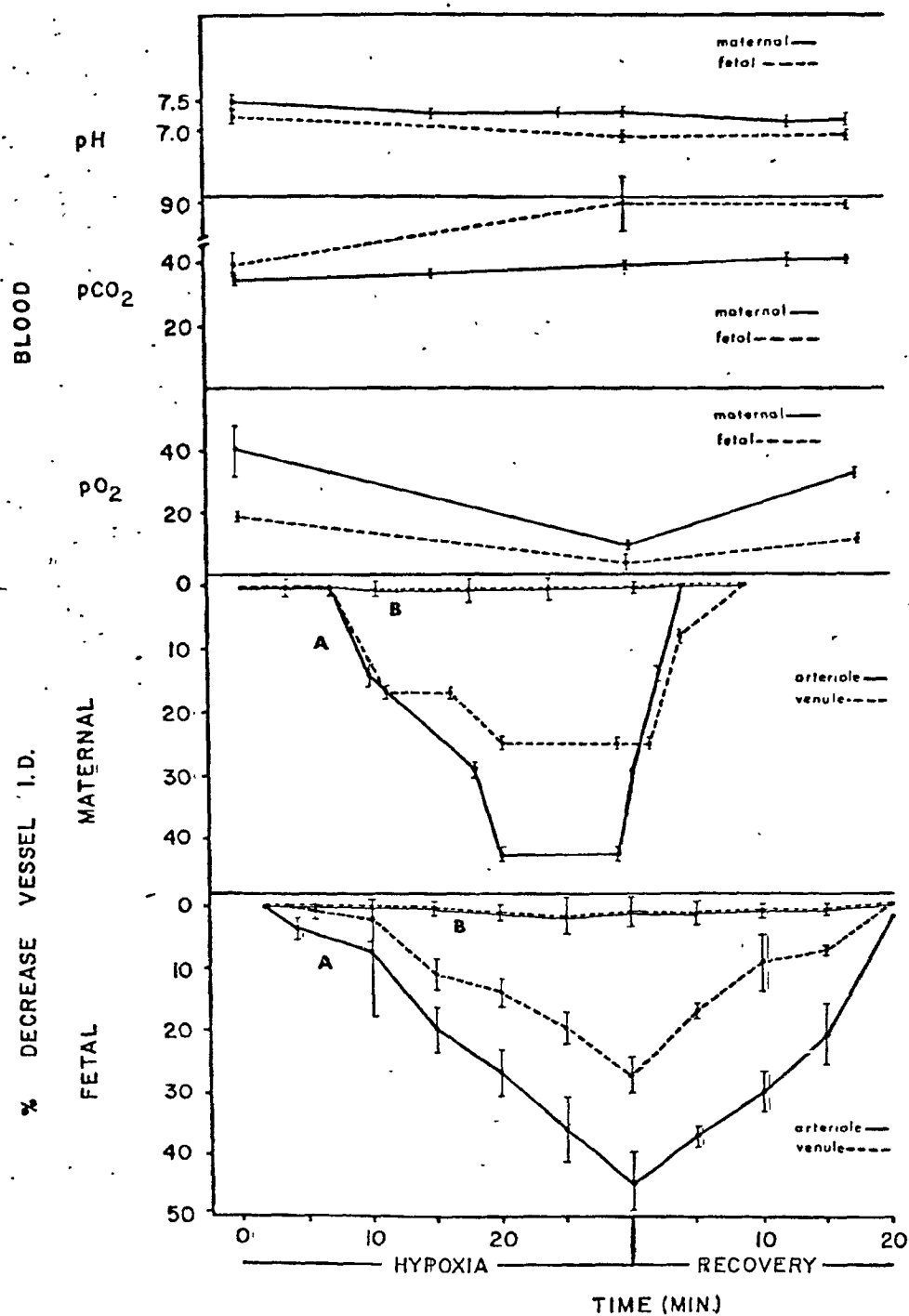
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transilluminate through them. This entails the use of a condenser in the optical system. I have in my laboratory a used Leitz Panphot binocular microscope, without optics. This microscope can be adapted for vital microscopy which then can be used for direct in vivo observations of tissues or organs using transmitted or reflected light or alternatively, television microscopy as mentioned in one above. Thus, the part of the experimental protocol which requires examination of organs such as liver in rats can be accomplished. (I have included a reprint, Microvas. Res. 3: 354-360, 1971, which will illustrate the methodology used for television microscopy and how it can be used to study living organs and tissues in situ).

3. Low Light Level Television Camera:

As mentioned in two above, when transilluminating through thick tissues or organs using either a quartz rod or a focusing condenser on a microscope, the amount of light passing through the specimen is greatly reduced from that which would pass through a 10 μ thick histologic slide. Thus, the conservation of light becomes imperative. To help offset this loss of light, a higher intensity light source can be used in conjunction with a television system which can provide useful pictures under compromised lighting conditions. The low light level Cohu television camera (2850 series) containing a silicon diode-array vidicon can be used in such conditions. The automatic light range controls are fully operational for scene brightness changes from 0.5 footlambert to 25,000 footlamberts with an f1.4 lens. After seeing a demonstration of this camera, I am convinced of its applicability to television microscopy under compromised lighting conditions and of its superiority over the two vidicon cameras I presently have in my laboratory.

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MATERIALS AND METHODS

One-hundred and fifty male Sprague-Dawley rats (100-125 g) were used. Rats in groups of 25 were injected intraperitoneally or subcutaneously with 0.15 cc CCl₄ (carbon tetrachloride) mixed with 0.15 cc of mineral oil every other day for a period of 2 weeks. Control animals were given placebos of mineral oil or of Ringer's solution. The animals were fed a standard laboratory diet and were given water *ad libitum* throughout the course of the experiment.

An *in vivo* method reported by McCuskey (4,5) was used to study the liver. Animals were anesthetized by intraperitoneal injection of 20% ethyl carbamate (Urethan, 1.5 g/kg). After laparotomy a lobe of the liver was exteriorized by floating it onto a window of Saran Wrap (Dow Chemical, Midland, Michigan) which overlaid a substage condenser. Homeostasis was maintained by irrigating the surface of the liver with Ringer's solution warmed to body temperature (4,5). Transillumination of the exposed edge of the liver was accomplished with monochromatic light (390-650 mμ) brought to the liver by the substage condenser of a modified Leitz Panphot microscope. Observations were made by direct microscopy at magnifications of 100-1000× using Leitz water-immersion objectives (10, 22, 50, and 80 ×) with appropriate oculars, or the optical images were projected onto the photocathode of a RCA vidicon (PK-301) or image orthicon television system (TK-31A) and kinerecorded with a modified Arriflex-16S, 16-mm motion picture camera. Kodak 16-mm Tri-X reversal film was used (4,5). During the 2-week treatment period, the liver was examined immediately after CCl₄ administration and at intervals up to 2 weeks.

Routine histological frozen and paraffin sections were prepared from the liver from some of the animals in order to correlate the *in vivo* microscopic observations with fixed tissue sections; these were stained with oil red O or hematoxylin and eosin.

RESULTS

The structure of the liver was altered progressively during the 2-week period of treatment with CCl₄. Treatment and observations could not be extended past 2 weeks since the CCl₄ induced widespread necrosis, shrinkage, and thickening of the liver preventing adequate definition of the histology of the organ *in situ*.

Carbon tetrachloride had its first visible effect on the microvasculature within 2 hr. This effect became progressively more severe during the first 2 days of treatment. In the majority of the livers observed during this period, the endothelium of the sinusoids became thickened and white blood cells adhered to the walls of the sinusoids in the centrilobular portions of the lobules (Fig. 1). Small aggregates of leukocytes also were observed to adhere to the endothelial wall of central venules. In the healthy animal with optimal circulation, white blood cells were never observed to adhere to the endothelium in this manner (Fig. 2). This diffuse sticking and aggregation resulted in stasis and congestion in the sinusoids, and led to an apparent reduction in the linear velocity of blood flow in the central venules as compared with observations made in control animals. Many of these white cells also passed through the endothelium of the central venules and sinusoids and entered the extravascular space. Subsequently, in many

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LOUISIANA STATE UNIVERSITY MEDICAL CENTER

1100 FLORIDA AVENUE • NEW ORLEANS, LOUISIANA • 70119

DEPARTMENT OF ANATOMY

June 19, 1973

Frederic W. Nordsiek, Ph.D.
Associate Scientific Director
The Council for Tobacco Research- U.S.A., Inc.
110 East 59th Street
New York, New York 10022

Re: Your Grant Application #912

Dear Dr. Nordsiek:

Please accept my apologies for the omission of the signature of our Comptroller from my grant application. Enclosed is a xeroxed copy of the budget page (p. 28) with Mr. Pohlig's signature.

My delay in replying to your letter was due to my visitation to several laboratories in Scandinavia during May and part of June. While visiting the Department of Experimental Medicine at Pharmacia, AB, in Uppsala, Sweden, I learned a fluorescent technique to study the microcirculation which they have been using for a couple of years. After learning this methodology, I'm convinced of its applicability in studying the permeability of blood vessels under normal or pathologic conditions. In the research grant submitted to the Council for Tobacco Research, I stated that the results from previous experiments in my laboratory on the effects of carbon monoxide (CO) on the fetal or adult mesenteric microvascular system suggest that CO alters the permeability of capillaries or post-capillary venules. The methodology used to study these changes in permeability can easily follow any alteration in the behavior of the cellular elements of blood; however, it is more difficult to document in vivo the passage of plasma constituents across the endothelium which probably precedes any cellular passage due to the effects of CO. This fluorescent technique can be used to study any increased endothelial permeability to plasma which will provide additional information to the changes which have been described in the behavior of blood cells after CO exposure.

In order to examine the effects of CO on the permeability of the fetal and adult microvascular system to plasma constituents, fluorescein conjugated dextran (M. W. 145,000) can be administered I.V. in a 5% Ringer's solution (200 mg./kg.) which is iso-oncotic. The presence of the

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13. Budget: (1st year)

A. Salaries (Personnel by names)

Professional

Samuel G. McClugage, Jr.
Marilyn L. Zimny

% time

50%
15%

Amount

REDACTED

Technical

*Research Assistant (including fringe benefits) 100%
*Research Assistant (including fringe benefits) 50%
Secretary (including fringe benefits) 50%

REDACTED

Sub-Total

REDACTED

B. Consumable Supplies (list by categories)

100 Pregnant rabbits @\$20.00
25 Non-pregnant rabbits @\$10.00
Anesthetic gases (CO₂/Air, O₂)
Motion Picture film and film processing
Misc. supplies (chemicals, surgical instruments, etc.)

2,000.00
250.00
400.00
1,000.00
600.00

Sub-Total

4,250.00

C. Other Expenses (itemize)

Animal Care (.20/day/rabbit)
*100 hours of use of Scanning Electron Microscope @\$20.00/hr.
Travel (for two people to attend one meeting per year)
*Machinist expenses

750.00
2,000.00
600.00
400.00

Sub-Total

3,750.00

D. Permanent Equipment (itemize)

*Monochromatic system adapted for quartz rod
*Optical equipment necessary to adapt Leitz Panphot Microscope
for in vivo microscopy
*Low light level Cohu television camera including
sync. generator

2,150.00
4,790.00
4,750.00

Sub-total

11,690.00

E. Overhead (15% of A + B + C)

Overhead
Total

3,444.00

REDACTED

Estimated Future Requirements:

	Salaries	Consumable Suppl.	Other Expenses	Permanent Equip.	Overhead	Total
Year 2	REDACTED	4,250.00	3,350.00	1,400.00	3,504.00	28,262.00
Year 3		4,250.00	3,350.00	900.00	3,601.00	28,506.00

Salaries include increments of 6% per year plus 10% for fringe benefits

It is understood that the applicant and institutional officers in applying for a grant have read and found acceptable the Council's "Statement of Policy Concerning Conditions and Terms Under Which Project Grants Are Made"

* (See Justification of Budget on next page)

Signature *Samuel G. McClugage, Jr.*
Director of Project Samuel G. McClugage, Jr.
(504) 947-9961 - ext. 255 Telephone

Signature _____
Business Officer of the Institution E. F. Pahlig
Comptroller (504) 527-5142 Telephone

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lobules red blood cells extravasated through the endothelium of the sinusoids and central venules resulting in minute hemorrhages. Toward the end of the treatment period, central venules were obscured by centrilobular necrosis, hemorrhage, and moderate fibrosis.

While these changes produced marked alteration of blood flow, not all vascular channels were affected; some vessels and lobules were relatively normal in appearance. The lesions described were always most severe in the centrilobular areas while the portal areas of the same lobules were normal in appearance, exhibiting little vascular involvement.

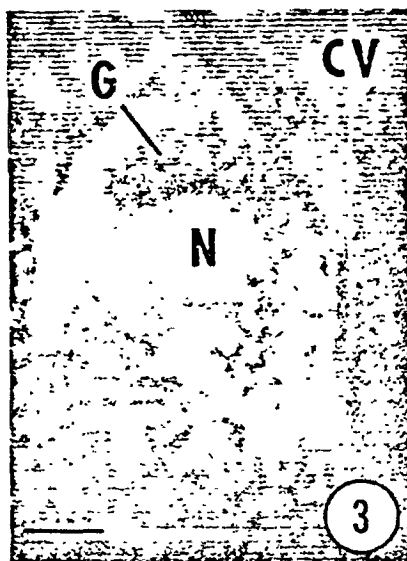


FIG. 3. Hepatocytes with perinuclear granulation (G), N, nucleus; CV, central venule. Five days after initial administration of CCl₄. Single frame from motion picture. Size marker is 5 μ .

Visible parenchymal alterations followed the microvascular response. During the first 4 days of exposure to CCl₄, small fat vacuoles developed in hepatocytes extending from the midlobular region to the central venules. These vacuoles displaced the nuclei to the periphery of the cell. Cells in the periportal areas exhibited no detectable fatty change.

During the intermediate stage of treatment, 5-9 days, fat accumulation became more severe but remained predominantly in the centrilobular region. Cells now contained multiple vacuoles; others exhibited perinuclear granulation, especially cells adjacent to central venules (Fig. 3). Fibrosis became evident in a few necrotic centrilobular areas.

In the later stages, 10-14 days, in addition to the above changes, large hypertrophied hepatocytes were evident in centrilobular and midlobular areas. The hepatocytes contained a mass of perinuclear granules surrounded by a homogeneous cytoplasmic matrix; this effect was a progressive development from the parenchymal changes

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flow in perfused livers, described by Rice and Plaa (11), could well have been caused by vascular lesions such as we observed rather than by obstruction from increased cellular triglyceride content in the early phase or later necrosis. Nakata and Higaki (7) reported that the hemodynamic changes always followed the appearance of parenchymal lesions in their perfused liver preparations. It would be of considerable interest to determine if the intravascular hemodynamic changes observed *in vivo*, i.e., adhesion and aggregation of white blood cells, can be induced in an organ which is perfused. Although the vascular lesions described in this report were not observed in other *in vivo* studies (14,15), this may be due to differences in methodology, especially the use of high magnifications in this work as opposed to low magnifications and poor resolution.

Although the process of fixation probably washed out many of the adherent white blood cells, tissue sections from this experiment did suggest areas of white cell adhesion and diapedesis through the walls of sinusoids and central venules. Some of the white cells within the vessels were enmeshed in a fibrin matrix.

The parenchymal changes observed *in situ* after CCl₄ poisoning follow conditions described by Gall (2) in cases of nutritional cirrhosis, namely, hepatocyte disintegration, focal necrosis, and a variety of cytoplasmic changes. Pseudolobule formation and fibrous interconnections were lacking probably because of the relatively low dosage of CCl₄ and the short treatment period. For the most part, hepatic architecture was maintained, but widespread centrilobular necrosis occurred in the later stages of treatment.

During the 2-week sequence of treatment, the hepatocytes appeared to undergo three progressive stages of morphologic alteration, i.e., fatty change, hydropic degeneration, advanced hydropic degeneration, and necrosis. In any one stage of parenchymal change the most advanced lesions were always nearest central venules. Thus in the final stage of treatment, at 10–14 days, the parenchymal lesions from within the center of the lobule outward to the periphery, mimicked the CCl₄-induced alterations described by other investigators using light microscopy, namely, necrosis, hydropic degeneration, and fatty changes. The most severe change was always farthest from the oxygenated blood supply (4,6).

The distribution of lesions observed *in vivo* as areas of fatty change, hydropic degeneration (balloon cells) (8), and necrosis was confirmed by the use of frozen tissue sections stained with oil red O.

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1003542269

"In Vivo" Microscopic Study of the Response of the Hepatic Microvascular System to Carbon Tetrachloride Poisoning¹

SAMUEL G. MCCLUGAGE, JR.,² AND ROBERT S. MCCUSKEY³

Department of Anatomy, University of Cincinnati College of Medicine,
Cincinnati, Ohio 45219

Received March 15, 1971

The initial effect of carbon tetrachloride poisoning on the microvascular system and parenchyma of the rat liver was studied using an *in vivo* microscopic method. The results suggest that the initial lesion is in the microvascular compartment; this reaction institutes an inflammatory response characterized by adhesion of white blood cells to the endothelium of sinusoids and central venules, and subsequent diapedesis of white blood cells. Carbon tetrachloride later induces alterations in the parenchyma resulting in fatty changes, hydropic degeneration, and necrosis in a stepwise manner. The combined events, microvascular and parenchymal, produce marked alterations of hepatic blood flow thus promoting anoxia and pathologic lesions.

INTRODUCTION

The pathogenesis of carbon tetrachloride-induced cirrhosis is in some dispute. Aterman (1) described cirrhosis induced by carbon tetrachloride as a chronic inflammatory process that produces alterations in the vascular and parenchymal components of the liver. Contributing features cited have included fatty changes, necrosis, and congested sinusoids (10-12). Petrelli and Stenger have suggested that the wall of the sinusoid might be the initial site of damage by carbon tetrachloride (9). On the other hand, Hase (3), using silicone rubber perfusions, studied the effects of carbon tetrachloride on the "microcirculation" (3) of livers in rats. He concluded that the microvascular lesions always followed the parenchymal lesions. Other investigators (14, 15), studying liver microscopically, reported the response of the exposed, intact liver to this hepatotoxin. Only limited observations could be made in these *in vivo* studies (14, 15) since relatively low magnifications were used with resulting poor resolution, thus prohibiting accurate evaluation of cellular detail. No work has been reported using *in vivo* microscopic methods that permit observations of cellular detail at the limit of resolution of the light microscope (4,5). Thus, the present study was designed to examine concomitantly the hepatic microvasculature and parenchyma in the living state in order to elucidate further the events that antecede cirrhosis.

¹ Presented in part in motion picture form at the Midwestern Association of Anatomists Meeting, Omaha, Nebraska, November 15, 1969.

² Present address. Department of Anatomy, Louisiana State University Medical Center, 1542 Tulane Avenue, New Orleans, Louisiana 70112.

³ Recipient of N.I.H. Research Career Development Award, AM-42,370.

and fetal blood pH, pO_2 and pCO_2 were monitored using an ultramicro blood gas analyzer (Instrumentation Laboratories, model 123-S1, 125A).

The effect of catecholamines on the mesenteric microvasculature of the fetus and adult was tested by local, topical application of epinephrine (10 μ g), and norepinephrine (10 μ g) both before and after application of phentolamine or propranolol.

The responses of the fetal mesenteric microvasculature to hypoxia and subsequent recovery were recorded cinéphotomicrographically and were compared with those in the maternal mesenteric microvasculature.

Results. The responses of the fetal mesenteric microvasculature to acute hypoxia in the mother were vasoconstriction, reduced flow in large arterioles and venules (100–300 μ i.d.), severely reduced flow in small arterioles and venules (less than 100 μ i.d.), and elimination of flow in most capillaries. These responses occurred within 30 min after the administration of 8% O_2 was initiated. Recovery occurred within 20 min after the removal of the low oxygen mixture (Fig. 1). Similar responses were observed in the maternal mesenteric microvasculature but the responses were seen within 20 min with a lag in the initiation of the vasoconstriction and with recovery within 5–10 min (Fig. 1). During recovery vessel diameter was restored in parallel with blood pO_2 while pCO_2 still was elevated and pH depressed.

The above responses could be mimicked by local, topical application of epinephrine or norepinephrine. Local topical application of phentolamine blocked the above vasoconstrictive responses caused by hypoxia (Fig. 1), epinephrine, or norepinephrine in both the fetal and maternal vessels, and occasionally resulted in a slight dilatation of these vessels. Propranolol, however, failed to block the vasoconstrictive response to hypoxia. Acute maternal hypoxia did not induce tissue edema nor did it lead to intravascular erythrocyte aggregation and sludging in the vessels examined.

Discussion. These data illustrate that the response of the fetal mesenteric microvascular system to hypoxia is vasoconstriction and

suggests that this response is mediated by an oxygen dependant, alpha-adrenergic mechanism since: (i) the response could be mimicked by the administration of epinephrine or norepinephrine and could be blocked by an alpha-adrenergic blocking agent, phentolamine, but could not be blocked by a beta-adrenergic blocking agent, propranolol; and (ii) vessel diameter returned in parallel with blood pO_2 while blood pCO_2 remained elevated and blood pH depressed. Thus, it would seem that recovery following hypoxia in the fetal microvasculature and reestablishment of blood flow through capillaries of the mesenteric tissue is not so much dependant on the blood acid-base balance and pCO_2 as it is upon blood pO_2 , a finding that is in agreement with the results of Godfrey (8).

At this time, however, it is not clear whether the vasoconstriction is due to reflex neural mechanisms initiated by chemoreceptors, is due to humoral mechanisms, e.g., elaboration of epinephrine from the suprarenal, or is possibly a direct effect of hypoxia on the vessel wall. While the existence of functional chemoreceptors and autonomic innervation in the fetus is not clear (1, 2, 9–13), several studies suggest the importance of catecholamine release from the suprarenal during the last half of gestation in the response of the fetus to stress (1, 2, 14). Unfortunately, there is little or no information concerning the sensitivity of the fetal systemic vascular wall to varying concentrations of oxygen in the blood. Studies on isolated adult vessels, however, indicate that hypoxia induces vasodilatation, except in the lung where vasoconstriction is the result (15). While vasoconstriction of pulmonary vessels in response to hypoxia also has been demonstrated to be a direct, local effect in the fetus (1), there is little information concerning such direct action in the fetal systemic vessels. In addition, the relative sensitivities of the fetal systemic vessels compared with the adult to varied oxygen concentrations have not been reported.

In this study the data suggest that the response of the maternal and fetal vessels to maternal hypoxia are equivalent. Both maternal and fetal arterioles constricted approx-

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12. Biographical sketch:

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BIOGRAPHICAL SKETCH

(Give the following information for all professional personnel listed on page 3, beginning with the Principal Investigator. Use continuation pages and follow the same general format for each person.)

NAME Cupshaw, Mont R.	TITLE Associate Professor	BIRTHDATE (Mo., Day, Yr.) REDACTED
PLACE OF BIRTH (City, State, Country) REDACTED	PRESENT NATIONALITY (If non U.S. citizen, indicate kind of visa and expiration date) REDACTED	SEX REDACTED
EDUCATION (Begin with baccalaureate training and include postdoctoral)		
INSTITUTION AND LOCATION	DEGREE	YEAR CONFERRED
Idaho State College--Pocatello, Idaho	B.S.	1960
Washington State U.--Pullman, Wash.	M.S.	1963
University of Iowa--Iowa City, Iowa	Ph.D.	1966
		SCIENTIFIC FIELD
		Pharmacy
		Pharmacology
		Pharmacology

HONORS

Who's Who in American Colleges and Universities--1960
Who's Who in the West--1973

MAJOR RESEARCH INTEREST

Developmental Biochemical Pharmacology

ROLE IN PROPOSED PROJECT

Principal Investigator

RESEARCH SUPPORT (See instructions)

Biotransformation of Drug Substrates in Human Fetal Tissues,
CRBS-2500
July 1, 1973-June 30, 1974--\$13,832 (Total Project Period), 15% time.
National Foundation (March of Dimes).

RESEARCH AND/OR PROFESSIONAL EXPERIENCE (Starting with present position, list training and experience relevant to area of project. List all or most representative publications. Do not exceed 3 pages for each individual.)

Associate Professor of Pharmacology, School of Medicine, University of Washington (1973-present)
Assistant Professor of Pharmacology, School of Medicine, University of Washington (1969-73)
Assistant Professor of Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo (1967-69)
Instructor in Biochemical Pharmacology, School of Pharmacy, State University of New York at Buffalo (1966-67).
U.S.P.H.S. Graduate Trainee, The University of Iowa (1963-66)
Research Assistant, Washington State University (1962-63)
Teaching Assistant, Washington State University (1961-62)
Registered Pharmacist, Idaho, Nevada (1960-present)
Publications: See Section A, Number 15, Item d (pp. 20-21).

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FIG. 1. Changes (\pm the standard error of the mean) in the internal diameters of vessels in fetal and maternal mesenteric microvasculature, and changes in pH, pO_2 , and pCO_2 of the fetal and maternal blood, during hypoxia and recovery in anesthetized rabbits: A, normal response to hypoxia and following administration of propranolol; B, response to hypoxia after administration of phentolamine.

imately 45% while venules constricted approximately 25%. The smaller degree of venular constriction suggests that these vessels may have less functional innervation, may be less sensitive to catecholamines released from the suprarenal, or may be less sensitive to low oxygen saturation of the blood. The most probable explanation, however, is that these vessels contain considerably less smooth muscle in their walls than do their companion arterioles and are less capable of vasoconstriction.

Summary. The effect of maternal hypoxia on the microvascular system of fetal and pregnant adult rabbits was studied. The response of the fetal mesenteric microvasculature to hypoxia was vasoconstriction, reduced flow in the large arterioles and venules, and severely reduced flow in most capillaries. This response occurred within 30 min after initiation of 8% O_2 ; recovery occurred within 20 min after removal of the low oxygen mixture. Similar findings were obtained in the maternal mesenteric microvasculature but the responses were more rapid, occurring within 20 min, with recovery within 5-10 min. The responses appeared to be mediated by an oxygen dependant, alpha-adrenergic mechanism since, during recovery, flow and vascular diameter were restored in parallel

with the blood pO_2 even though blood pH still was depressed and pCO_2 was elevated, and since the vasoconstrictive response could be blocked by phentolamine but not by propranolol.

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1003542322

Response of the Fetal Mesenteric Microvascular System to Maternal Hypoxia¹ (34277)

ROBERT S. MCCUSKEY, SAMUEL G. MCCLUGAGE, JR.,² THOMAS J. MOORE,
AND MARIAN L. MILLER
(Introduced by R. C. Crafts)

Department of Anatomy, University of Cincinnati, College of Medicine, Cincinnati, Ohio 45219

Several studies have been reported on general cardiovascular responses of the fetus to maternal hypoxia or anoxia. These were reviewed recently by Dawes (1) and Rudolph and Heymann (2). The specific response of the fetal microvascular system to maternal hypoxia, however, has not been reported due, in part, to the difficulty involved in examining these vessels directly *in vivo* with the light microscope while maintaining homeostasis. This poor understanding of the microvascular system has prompted a series of studies of these vessels *in vivo* in rabbit fetuses with their placental circulations intact (3-6). The present paper reports the effect of maternal hypoxia on the fetal mesenteric microvascular system.

Materials and Methods. The mesenteries of 50 fetal and 15 adult pregnant rabbits (New Zealand albino) were studied. Fetal preparations and adult preparations were studied independently since technical complications did not permit simultaneous microscopic observations of fetal and adult mesenteries. In both preparations pregnant rabbits were anesthetized with ethyl carbamate (Urethane, 1.5 g/kg). To study the fetal mesentery a fetus was exteriorized with its placental circulation intact on various days of gestation between days 25 and 32 (av gestation in the rabbit is 32 days) and the fetal mesentery was exposed surgically. Homeostasis was maintained by constant irrigation with Ringer's solution of the surface of the mesentery as well as the fetal body surface which was covered with gauze sponges. The temperature of the

Ringer's was maintained at the maternal body temperature by regulating heaters (3-6). In addition, the ambient air surrounding the fetus was maintained at 37.5° by a Sage "air curtain" with its controlling thermometer probe placed on the surface of the fetus. To study the mesentery of the pregnant adult rabbit, the uterus was displaced and a loop of bowel was exposed. Homeostasis was maintained as in the fetus.

Observations of the mesentery of the fetus or of the pregnant adult were accomplished by transillumination of the tissue with light conducted to the mesentery by a hollow, fused quartz-rod (7) and examination with a Leitz stereo-binocular microscope equipped with 2×, 4×, 8×, and 12× objectives and 12.5× and 18× oculars. Using these optics magnifications of 25-216× were obtained. Alternatively, a modified Leitz compound monocular microscope was used equipped with 10×, 22×, 50×, and 90× water immersion objectives and a 10× ocular to provide magnifications to 900×. Measurements of the internal diameters of vessels were secured with a calibrated micrometer disc in the oculars.

To study the response to hypoxia of the mesenteric microvasculature of the fetus and pregnant adult, the mother received a mixture of 8% O₂/92% N₂ gas for 30 min by means of a closed circuit anesthetic machine. Then the low oxygen mixture was removed and the animal was allowed to recover breathing room air. This procedure also was repeated in fetuses and mothers to whose mesenteries an alpha-adrenergic blocking agent, phentolamine (50 µg), or a beta-adrenergic blocking agent, propranolol (50 µg), had been applied topically. Maternal

¹ Supported by a grant from the Heart Association of Southwestern Ohio.

² NIH Predoctoral Fellow: 1 FO1 GM-33179.

1966, the Food and Drug Administration performed analyses on 53 samples of margarine, 18.9% contained DDT with an average concentration of 0.026 ppm. In 1967, 13% of 23 samples contained an average of 0.014 ppm DDT.¹⁰

In contrast, during 1970, 100 butter samples were analyzed, 23% contained DDT with an average concentration of 0.005 ppm. In 1971, 5% of 84 samples contained only trace amounts of DDT (J.R. Wessel, Food and Drug Administration, written communication, August 1972). These data support the hypothesis that consumers of margarine are more apt to be exposed to DDT residues than are those who eat butter. It is suggested that nursing mothers eat butter rather than margarine.

The biological variations in pesticide content of breast milk revealed in this study require that future sampling be more precisely defined than in the past. The very significant increase in total DDT content of hind milk as compared with fore milk was the most striking variation encountered. Also of importance was the di-

minishing DDT concentration with increasing age of the donor. This relationship is consistent with Kroger's observation¹¹ that DDT content appears to decrease with the increasing number of children nursed by the mother. Future work should specify fore or hind milk collections and include age- and parity-specific concentrations.

We wish to reiterate that we know of no demonstrated danger from DDT to breast-fed infants which would warrant giving up the known advantages of breast-feeding. Nevertheless, we do feel that DDT concentrations in human milk should be more widely investigated in different geographic, socioeconomic, and racial groups and that the various biological factors affecting DDT excretion in human milk receive attention.

Household uses of DDT were banned by the Department of Agriculture during the autumn of 1969. On June 14, 1972, the Administrator of the Environmental Protection Agency issued a ban on the general use of DDT which took effect on January 1, 1973.¹² In 1970, the last year for which data

are available, approximately 25 million pounds of DDT were used in the United States (B. Fielding, Environmental Protection Agency, oral communication, December 1972). Public health, quarantine uses, and a few minor crop uses of DDT are exempted from the general ban and are estimated to require a few thousand pounds of DDT annually. As of this writing, several industrial groups are suing to gain exemptions for certain other agricultural uses. The ban will go into effect while these suits are in progress, but should the exemptions be granted, usage is estimated to be about one half million pounds of DDT per year. The order also does not affect exports of DDT for use in other countries; therefore, significant amounts of this pesticide will continue to enter the earth's ecosystem.

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MISCELLANEOUS

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described in the intermediate stage. At this time hepatocytes in the periportal areas of many lobules had undergone some fatty change while the centrolobular areas of these same lobules were necrotic.

Throughout the course of the experiment, increasing deposits of fat, debris, and hemorrhage beneath the capsule made visualization difficult and at times impossible.

Frozen and paraffin sections confirmed the lesions observed *in vivo* in the microvascular and parenchymal compartments.

DISCUSSION

The hepatotoxicity of CCl_4 has been the subject of many investigations. The variability of results may be attributed to the different dosages of CCl_4 used, the different routes of administration, and the age and sex of the animal since all of these factors are known to affect the hepatotoxicity of CCl_4 (13). By using low doses of CCl_4 in conjunction with a short time sequence (2 weeks), it was possible to study in the living state some of the initial effects of CCl_4 upon the rat liver. The histologic alterations induced by CCl_4 and observed sequentially *in vivo* were the following: (a) endothelial damage with adhesion of white cells to the walls of sinusoids and central venules; (b) reduced blood flow through sinusoids and central venules as compared with control animals due to plugging of these vessels by adherent white blood cell masses; (c) diapedesis of white cells into the extravascular compartment; (d) fatty metamorphosis and hydropic degeneration of parenchymal cells, centrolobular necrosis, and hemorrhage; (e) further reduction of blood flow through sinusoids as a result of impingement of hypertrophied hepatocytes on the sinusoids; and (f) widespread vascular congestion and necrosis. These results suggested that the primary site of injury of the CCl_4 -poisoned rat liver might be in the microvascular system.

The alterations described above are not unexpected since CCl_4 -induced cirrhosis has been classified as a chronic inflammatory condition (1). The results of this study are in agreement with the findings of Zweifach *et al.* (16) who studied how damaged endothelium alters the behavior of white cells during an inflammatory response. The adhesion and aggregation of white blood cells to endothelium may be a cause of tissue anoxia by greatly reducing blood flow through the microvascular system of the liver. Plugging of sinusoids and central venules by leukocytes, diapedesis of white blood cells, and hemorrhage reflect endothelial damage (16). Apparently this damage is initially induced by the CCl_4 , and later augmented by anoxia due to a decreased blood flow through the sinusoids. Petrelli and Stenger (9) were able to reduce the hepatotoxic effects of CCl_4 by giving trypan blue prior to the CCl_4 . The trypan blue increased the cytoplasmic mass of the lining cells of the sinusoid, thus reducing the accessibility of the extravascular compartment to CCl_4 . This suggests, as does this report, that the initial lesion is in the microvascular compartment. In addition, subsequent swelling of parenchymal cells further reduced blood flow. Since the centrolobular areas are farthest from the oxygenated blood supply, they would be affected first by the anoxia (4,6); the most severe parenchymal lesion was always in the centrolobular region.

Rice *et al.* (10) thought that the initial lesion in CCl_4 hepatotoxicity was not vascular. They believed that blood flow played a minor role in furthering the damage once the parenchymal lesion had been induced. However, an increasing biphasic resistance to

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the number of days per week the donor ate meat or fish. However, when those women who used butter and margarine were compared (Table 3), those using margarine had a higher concentration of total DDT in their milk than did those who used butter ($P < .04$).

While there was no correlation with the infant's age at the time the milk sample was donated, there was a negative correlation between the mother's age and the total DDT concentration in her milk, i.e., the older the mother, the lower the concentration tended to be.

Twenty-four matched pairs of milk samples were obtained. Milk from a full breast (fore milk) was compared with milk from a nearly empty breast (hind milk) from the same donor at the same feeding. The two sets of samples showed a striking difference (Table 4): the total DDT concentration was significantly higher in the hind milk ($P < .01$).

We sought to determine the dependence of milk DDT concentration upon the date of sample collection. These preliminary results suggest a seasonal dependence of total DDT concentration, with DDT concentration in the late summer ranging up to 60% (0.08 ppm) higher than DDT concentration in the latter part of the winter. Additional specimens obtained over an extended period of time will be needed to verify this seasonal periodicity.

Comment

Organochlorine pesticides are now universal pollutants; they can be detected in virtually all animal tissues, even those sampled in remote parts of the earth far from areas of large-scale pesticide use.⁹ It is now accepted that low tissue concentrations of such pesticides may produce subtle injury to species of birds, fish, and other nontarget organisms.¹⁰ Concern over the potential effects of pesticide residues in man has led to extensive routine food-monitoring programs in this and other countries and upper limits of acceptable pesticide concen-

trations have been set for many food items including milk.

The monitoring of man has been considerably less extensive and less standardized; milk is a pertinent example. The World Health Organization (WHO) has set a practical residue limit for total DDT in cow's milk of 0.05 ppm.¹¹ The Food and Drug Administration uses this value as the maximum permissible concentration of total DDT in the regular monitoring of commercial cow's milk shipped in interstate commerce. The recent public controversy regarding breast milk apparently emanates from newspaper reports of the higher concentrations of DDT in human milk.³ As has been noted, the data base supporting these reports is, in comparison to that for cow's milk, extremely small. Nevertheless, it and the results of the current study do support the general conclusion that human milk contains a higher concentration of total DDT than does cow's milk.

The higher concentration of DDT in human milk is not an unexpected finding. Pesticides tend to become more concentrated as one samples up a food chain¹²; that is, meat-eaters (including man) store more DDT in their tissues than do herbivores, such as cattle. Hence, human milk would be expected to contain more DDT than that from cows.

The mean concentrations of total DDT in all seven geographical areas sampled in this study were in excess of the WHO upper limit for cow's milk. This was also the case in the two recent publications that reported on samples from various parts of Pennsylvania¹¹ and Canada.³

The WHO maximum admissible daily intake of DDT is set at 0.01 mg/kg of body weight.¹¹ Thus, a 4 kg infant ought not ingest more than 0.04 mg of DDT per day. If an infant drinks approximately 650 ml of milk per day,¹³ the milk must contain less than 0.06 ppm DDT if the WHO limit for cow's milk is not to be exceeded. The mean in our study was 0.17 ppm.

It is imperative to state at this point that we know of no demon-

strated damage to breast-fed infants from DDT. Furthermore, the study of Hayes et al.¹⁴ indicates that adult men are not injured directly by prolonged high-level oral doses of DDT. Increased mortality among neonatal rats nursing very heavily DDT-treated mothers has been reported,¹⁵ but the relevance of that study to man is conjectural. The absence of a direct connection with illness notwithstanding, it appears prudent to monitor human breast milk for pesticide content.

Both biological and environmental factors correlating with the concentration of DDT in breast milk were revealed in this study. Although the sample size is among the largest in the literature, it is still small and the results are regarded more as an impetus to further study than as a definitive investigation.

The lower DDT content in the milk from the Long Island communities suggests that there may be significant variation with geographical area. We have no explanation for this observation.

Pesticide exposure was a less clear correlate than expected. Large-scale exposure in the past was not associated with increased DDT content, nor was personal home use of pesticides. The employment of exterminators seemed to be protective, however: it is known that commercial operators rarely use organochlorine pesticides in dwellings. The suggestion of a seasonal influence on milk DDT concentration might be due to environmental factors such as seasonal changes in diet or changes in domestic or garden use of pesticides.

Eating margarine rather than butter was associated with higher DDT concentrations. While we are reluctant to imply a directly causal relationship with this single dietary item, it is of interest that margarine is made largely of cottonseed oil and that DDT has been used extensively in the cotton industry.¹⁶ Some direct measurements of DDT residues in margarine and butter have been made: during the period from 1964 to

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DDT Concentrations in Human Milk

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Human milk from seven US cities was analyzed for total DDT (DDT plus DDE) content. The mean of 138 samples was 0.17 ppm (range, <0.02 to 0.83 ppm) which is in excess of the World Health Organization's recommended maximum concentration in cow's milk (0.05 ppm.)

Use of commercial exterminators was associated with lower DDT levels than was personal home use of pesticides; donors using butter had lower concentrations than those using margarine. DDT levels diminished with increasing maternal age and milk obtained after nursing contained significantly more DDT than milk obtained at the start of nursing.

While no adverse effects to infants due to DDT in human milk has been documented, systematic monitoring of DDT and other environmental pollutants in man is needed.

Concern has been expressed in both the scientific literature^{1,2} and the lay press³ over the concentrations of DDT and its metabolites in human milk. This has resulted in some worry to women breast-feeding or planning to breast-feed infants.

The magnitude of public discourse has been somewhat disproportionate to the extent of the data. The number of pesticide residue determinations in human milk is small and the colorimetric methods employed in earlier

work are open to some question.⁴

Only eight articles have appeared in the English language literature over the 27 years, 1945 to 1972—a period of great change in the extent of pesticide use. As reviewed by Ritcey et al,⁵ seven additional studies have been published in the USSR and 12 in European countries. At present, the data are not sufficient to delineate geographic, racial, socioeconomic, and other possible variations in DDT concentrations in human milk. Preliminary data bearing on some of these questions are presented here.

Materials and Methods

Samples of human milk were obtained from white, urban, middle-class donors residing in several towns on Long Island and in Rochester, NY; Chicago; Lexington, Ky; Nashville and Memphis, Tenn; and Los Angeles. Samples were obtained during the period from June 1970 through October 1971. Donors also completed a brief questionnaire regarding their exposure to pesticides, food habits, and weight gain or loss. Samples were kept frozen in polyethylene bags or in glass bottles until analysis. Chlorinated hydrocarbon pesticides were extracted from these samples by the method described by Schafer et al.⁶ Ten milliliters of the milk was saponified with potassium hydroxide solution (25% solution of potassium hydroxide) and then extracted with 10 ml of hexane. The hexane extract was then sealed in a glass ampule until analysis. This method of sample preparation quantitatively converts DDT to DDE⁷ and results will be expressed as concentrations of total DDT.

Quantification of DDE was achieved with a gas chromatograph equipped with an electron capture detector, field-emission

type, or with another gas chromatograph also equipped with a radioactive nickel electron capture detector (⁶³Ni). The field-emission electron capture detector (ECD) was calibrated with standard aliquots (2 to 10 ng) of DDE (Varian Associates Nanogen Pesticide Standards) in benzene which also established the linear range of this ECD. Each analysis of 10 μ l to 20 μ l portions of the hexane extracts was followed by an injection of the analytical standard (DDE) to compensate for the drift of the field-emission ECD. The ⁶³Ni ECD was calibrated with several aliquots of the analytical standard (0.05 to 0.20 ng DDE); a standard was also injected after each sample analysis to insure reliability. Samples which indicated high levels of DDE on first analysis were diluted 1:10 so that a 1 μ l to 2 μ l injection would deliver a quantity of DDE known to be in the linear range of detection to the ⁶³Ni ECD.

Sample analyses were equally well accomplished on a 2 meter \times 3 mm column of 10% DC-200 on Anakrom ABS 80/90 mesh support at 197 C (also used at 210 C) and a 2 meter \times 3 mm column of 3% Dex 300 on Chromosorb G-HF 80/100 diatomite mesh support at 215 C. Two other diatomite support columns were used to confirm the identity of DDE in selected samples: a 2 meter \times 4 mm column of mixed 5% QF-1 and 5% SE-30 on Chromosorb W60/80 mesh (acid washed) and a 2 meter \times 4 mm column of 25% SF-96 on Chromosorb W60/80 mesh (acid washed).

Blank analyses were run on distilled water samples to establish that reagents were not introducing spurious results. Preliminary studies using commercial cow's milk demonstrated that the plastic bags used for storage did not contaminate the samples. Analyses of fresh milk and milk which had been stored frozen in plastic bags for two months showed no discernible differences.

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